Degradation of indigoid compounds by *Micrococcus* sp.

By

Chun-fai Lai

Thesis submitted as partial fulfilment for

the degree of Master of Philosophy

July, 1991

Division of Biology

Graduate School

The Chinese University of Hong Kong

Thesis Committee

Dr. K.K. Mark (Supervisor)

Prof. S.T. Chang

Dr. P.K. Wong

Dr. K.H. Yung (Head,

Division of Biology, Graduate School)

Prof. Dickson Liu (External Examinar)

ACKNOWLEDGEMENTS

I wish to express my most sincere gratitude to my supervisor, Dr. K.K. Mark for his excellent guidance, encouragement and enthusiasm in the past two years.

I would also like to thank the members of my thesis committee, Prof. S.T. Chang, Dr. K.H. Yung, Dr. P.K. Wong and Prof. Dickson Liu for their valuable advice and criticism.

I am grateful to Mr. C.L. Cheung and Mr. K.O. So for their discussions and technical assistance.

Finally, I wish to express my deepest gratitude to my family for their continued support and encouragement during my years of study.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

A_{609}	Absorbance at 609nm
A_{650}	Absorbance at 650nm
DOC	Dissolved Organic Carbon
I.M.	Isolation Medium
NMR	Nuclear Magnetic Resonance
TLC	Thin Layer Chromatography

ABSTRACT

The use of various dyestuffs has brought essential colours into our lives. However, at the same time, the dyestuffs also cause serious environmental pollution. Biodegradation is a newly developed approach besides physical and chemical methods to counteract the above problem. Nevertheless, the present studies on dyes biodegradation are mainly focused on azo dyes while other dyes such as the indigoid dyes are neglected. Indigo is a dye commonly used in textile industry for denim products. Yet, there is not a single report about its biodegradation for the past 20 years. In this study, indigo carmine is used as a model compound for the analysis of the biodegradation of indigoid dyes.

A number of strains had been isolated from the industrial area seriously contaminated with dyestuffs. These strains were able to decolorize indigo carmine on screening plate of the Isolation Medium (I.M.). Among them, six strains with the highest decolorization ability were identified. They included three *Bacillus* species, two *Micrococcus* species and one *Agrobacterium* species. *Micrococcus* sp. H-12 was chosen for further detailed characterization.

The decolorization ability was found to be inducible by the presence of indigo carmine. *Micrococcus* sp. H-12 had optimum growth and decolorization at the temperature of 37°C and pH of 6.5. Although the growth of strain H-12 in static culture was slower than in the shaking culture, the decolorization ability in static culture was much higher in terms of specific activity. At the peak of activity, the difference was six times higher in the static culture.

Strain H-12 had a better metabolic ability on complex carbon sources over the organic salt. Growth and decolorization of indigo carmine were the best when tryptone was supplied as the carbon source while growth was limited in the cases of acetate and citrate. Although indigo carmine did not seem to be able to serve as carbon source, it could promote growth when ammonium chloride was removed from the I.M. medium.

Indigo carmine was found to have minimum inhibitory effect to strain H-12. Substantial growth could be maintained in a concentration of indigo carmine up to 500 ppm.

The resting cells of strain H-12 were also characterized. The decolorization ability was the highest when the incubation temperature was 37°C and pH was 4.0. There was no great difference between shaking and static condition during incubation.

The degradation products of indigo carmine by strain H-12 had been isolated. There were two spots detected on the TLC plate under UV light. One of them was a major component and was purified by silica gel column. This purified component was analyzed by nuclear magnetic resonance (NMR) and mass spectrometry. Although there was not a conclusive chemical structure, the results indicated that this component was probably an aromatic compound with molecular weight of 149. This suggests that the degradation mechanism may involve a cleavage of the carbon-carbon double bond in the centre of the molecule.

LITERATURE REVIEW

I. Introduction

Colour seems to be indispensable to human life. The use of colorants can be traced back to prehistoric time. Cavemen decorated their walls with fascinating colours. Ancient Egyptians, Greeks and Romans had tried great efforts to produce different colours from various sources. Until the end of the nineteenth century, all the colorants were derived from natural sources. The majority were of botanical origin. The sources included plants, trees and lichen.

William Henry Perkin's discovery of the first artificial dye, mauveine in 1856 was really a great breakthrough in dyestuff industry (Gordon and Gregory, 1983). He derived it from aniline. Within one year, the manufacture was put in a commercial scale. Since then, with the light shed by the development of organic chemistry, there was a vigorous study about the synthetic organic colorants. In the third edition of Colour Index (Allen *et al.*, 1971), the number of dyes and pigments was nearly 40,000 and the number would continue to increase in the future.

II. Classification of dyes

With the vast number of dyes, there is no single classification system that is comprehensive enough. Nevertheless, the dyes can be generally classified according to their uses or chemical structures. If the dyes are classified by the chromophores, about ten to twenty dyes classes can be classified. Chromophores refer to the part of

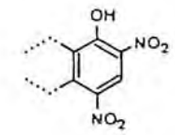
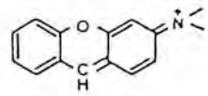
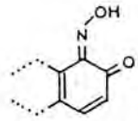
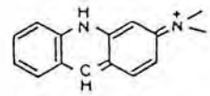
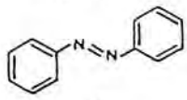
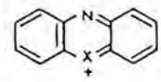
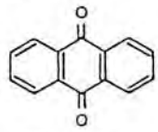
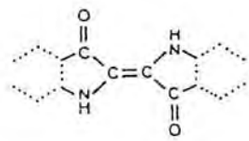
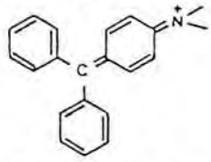
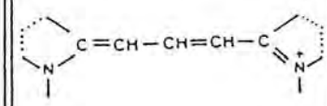
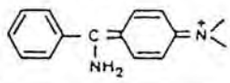
Chromophore	Dye Class	Chromophore	Dye Class
	Nitro Dyes		Xanthenes
	Nitroso Dyes		Acridines
	Azo Dyes		Quinoneimines
	Anthraquinones		Indigoids
	Triphenylmethanes		Cyanines
	Diphenylmethanes		

Figure 1: Classification of synthetic organic colorants by chromophores.(Meyer, 1981)

the chemical structure responsible for the characteristic colour of the dyes. Figure 1 has showed the major classes of dyes. Chromophores are usually involved in the biodegradation of dyes but whether they play a key role in biodegradation is not certain.

III. Adverse effects of dyes in the environment

The impact of pollutants to our environment has been a concern for long times. Many different systems have been studied for more efficient and effective measurement and control of the pollutants. In 1979, U.S. Environmental Protection Agency had published a list of 129 priority pollutants (Keith and Telliard, 1979) which became an important guildline for the industrial wastewater discharge. The environment impact of textile wastewater had been subjected to thorough assessment (Goodson, 1979; Gallup, 1979). In a survey of 151 dyes, seven priority pollutants were found to be present in amounts over 0.1% including three metals and three biocides in 31 dyes (Horning, 1979). In 1988, another list of colorants which classified as toxic on the basis of acute peroral LD_{50}, according to the criteria of the EEC 6th amendment, was released (Anliker *et al.*, 1988).

A. Release of dyes into the environment

Dyestuffs are discharged into the environment during production and processing as well as disposal of the used products. In the textile industry, over 10% of total amount of dyes is estimated to be lost during production and processing. In 1978, the total loss of textile dyestuffs was about 43,000 tonnes. This huge amount

of dyestuffs pollutants had found their way into our environment.

B. Toxicity to fish and mammalians

The majority of dyes on sales had been tested for toxicity to fish. They were found only slightly poisonous. In a survey of over 3,000 commercial dyestuffs, about 98% had LC_{50} values greater than 1 mg/l. Of the 2% of the dyestuffs with LC_{50} values smaller than 1 mg/l, 27 different chemical structures were found including 16 basic dyes and 10 triphenylmethane dyes (Holme, 1984). Little and Lam had conducted another study on *Primephales promelas* and showed that the LC_{50} was above 180 ppm for 29, between 1 and 180 ppm for 15 (Anliker, 1977). Only two out of the forty-six dyes being tested had a LC_{50} below 1 ppm. They were methyl violet (C.I. Basic Violet 1) and malachite green (C.I. Basic Green 4) with LC_{50} of 0.05 ppm and 0.12 ppm respectively. These two dyes were cationic dyes which constituted the majority of dyes toxic to fish. The cationic dyes (benzidine dyes and triphenylmethane dyes) was found to be almost as toxic as pesticide to freshwater fish. Different species of fish did not generally exhibit major difference in the sensitivity to the dyestuffs.

The factors for the assessment of the toxicological risks of dyestuffs include : the total exposure potential, the seriousness of the toxic effect and the chance of occurrence. Studies of the adverse effects to dyes to mammalian indicated that the dyestuffs are of generally low acute toxicity with LD_{50} seldom under 250 mg/kg. Anliker *et al.* (1988) had published a list of toxic colorants based on the acute oral toxicity test on rat. There were 12 toxic dyes with LD_{50} under 250 mg/kg. Among the numerous dyes, the benzidine dyes could cause chronic toxicity to man. Benzidine

and some of its derivatives were found to be carcinogenic (Meyer, 1981). Some benzidine-based dyestuffs were found to cause preneoplastic hepatic lesions in rats. Contact dermatitis or skin sensitization effects had been also reported for dyestuffs industry worker of some specific dyestuffs as well as consumers after exposure to dyed fabrics (Clarke and Anliker, 1980).

C. Toxicity to microorganisms

Inhibitory effects of dyes to microbial activity and growth usually exist only at particularly high concentration. However, some cationic dyes, mostly triphenylmethanes are inhibitory to microbial populations even at a low concentration. Various studied had been conducted to test the inhibitory effects of various dyes to different microorganisms (Fung and Miller, 1973; Michaels and Lewis, 1985; Ogawa *et al.*, 1988).

Fung and Miller (1973) had tested the effects of 42 dyes on the growth of 30 bacteria on solid medium. The results indicated that many available dyes were inhibitory to specific bacterial groups. In the study about the toxicity and sorption of five azo and triphenymethane dyes to freshwater microbiota by Michaels and Lewis (1985), some were to be highly toxic. Among them, basic violet 3 which had long been used as inhibitory agents in microbiology, was the most toxic with a mean survival rate of $20.7 \pm 6.57\%$.

Inhibitory effects of dyes to the activated sludge microorganisms had also been determined (Yonezawa and Urushigawa, 1977). A number of azo compounds had

been assessed in the study. The results showed that some of them were inhibitory and aminoazobenzenes exerted a strong inhibition effect. The inhibition effect of these azo compounds on activated sludge microorganisms was suggested to be related to the cell membrane permeability.

In another study performed by Brown *et al.* (1981), the inhibitory effect of the dyestuffs on the activated sludge was assessed by the measurement of the respiration rate. This large scale screening test had included dyes from various dyes classes e.g. azo, disazo, polyazo, anthraquinone, triarylmethane, oxazine, xanthene and phthalocyanine. Among the 202 dyestuffs tested, about 10% showed an inhibitory effect in such a concentration that likely to reach a sewage treatment plant. Of these dyestuffs, 18 showed an IC_{50} of less than 100 mg/l including 3 with an IC_{50} of between 1-10 mg/l.

However, some studies indicated the possibility that the inhibitory effects could be significantly lowered by acclimation (Idaka *et al.*,1985; Ogawa *et al.*,1981).

D. Colour contamination

Besides the toxicity to living organisms, the colour imposed by the dyestuffs are often objectionable to the public. The coloured wastewater would normally be observable at the level of 1 mg/l. This level is unacceptable for natural water in asethetic grounds. Different dyestuffs are easily deposited on the sediment of water courses. This situation is especially serious in the case of insoluble dyes. The colour contamination may hinder the penetration of light into the water and hence cause

adverse effects to the aquatic life.

IV. Dyes removal by physical and chemical treatment processes

Untreated effluent is frequently discharged from the dyestuff factories. Chung *et al.* (1981) had tested for the mutagenicity of 17 commercial used dyes and 16 of their metabolites or derivatives by the *Salmonella*-mammalian microsome mutagenicity test. Many of them were found to be mutagenic. In addition to the toxic substances, the effluent is highly coloured and objectionable. The effluent thus, has caused a severe environmental problem and more efficient and effective treatment processes are in urgent need.

Conventional physical and chemical treatment processes to tackle the dyes pollution include adsorption on activated carbon, resin adsorption and ion exchange, precipitation and flocculation using lime and alum, and ozonation (Davis, 1991; Netzer *et al.*, 1976).

Activated carbon seems to be suitable for removal of acid, basic and reactive dyes. However, a significant portion of the pore space was proved to be inaccessible to individual dye molecules. (Tarasevich *et al.*, 1988). In addition, activated carbons are costly materials. Ion-exchange resins have been used to remove anionic and cationic dyestuffs. However, dyestuffs mixture recovery is normally not economic and the disposal of the desorbed materials would impose additional cost. Chemical precipitation are capable to remove certain dyes. Nevertheless, the chemicals are expensive in long-term running and the dosage must be carefully monitored to avoid

additional chemical pollution. Of the chemical processes for dyes removal, ozonation has obtained the greatest practical success. However, a large amount of ozone is necessary in order to achieve an extensive degradation of the dyestuffs and so the process would become too costly for large scale operation.

More advanced treatment processes are actively investigated. These methods includes adsorption using new materials such as maize cob (El-Geundi, 1991), electrochemical treatment method in the presence of chlorine ions (Yakovlev *et al.*, 1988), chemical precipitation using aniline and formaldehyde (Filippov *et al.*, 1988) and τ-radiation induced oxidation of dyestuffs. These methods are still in the experimental stage but have not obtained any practical significance yet.

V. Biodegradation as anti-pollution measure

As we can see, physical and chemical treatment process usually require a high operational cost both in terms of materials and energy consumption. The disposal of the concentrated pollutants may impose another problem. Furthermore, these abiotic methods are not effective in such a low pollutants concentration as normally found in the sewage. Biodegradation of chemical wastes using microorganisms seems to be a potential and promising alternative (Ghisalba, 1983). There are numerous reviews to discuss the possibility of the use of microorganisms in cleaning our environment (Alexander, 1981; Atlas, 1981; Finn, 1983; Kobayashi and Rittmann, 1982; Patterson and Kodukala, 1981; Wallnöfer and Engelhardt, 1984). Studies have been carried out about the biodegradability of the pollutants compounds (Tabak *et al.*, 1981; Robra, 1986) and the correlation between biodegradation rate and the chemical structure as

well as the chemical concentration and environment (Lee *et al.*, 1980; Hicks *et al.*, 1990; Painter and King, 1985; Wang *et al.*, 1985). The isolation of the desirable degrading strains becomes a popular interest (Cook *et al.*, 1983; Leisinger, 1983).

Treatment of dyeing waste from factories is a major concern of the environment microbiologists (Straley, 1984). As there are few reports about the accumulation of large quantities of dyestuffs in the global environment, it seems very probably that microbial removal of dyestuffs by biodegradation exists. With much efforts devoted, many different microorganisms have been isolated to degrade a number of dyes (Bumpus and Brock, 1988; Chung *et al.*, 1978; Cripps *et al.*, 1990; Glenn and Gold, 1983; Idaka *et al.*, 1978; Kulla, 1981; Kwasniewska, 1985; Ogawa *et al.*, 1988; So, 1989).

A. Removal of dyes by activated sludge

Dye removal by activated sludge is primarily elicited by adsorption onto the biological sludge solid. However, biodegradation also exists. Shaul *et al.* (1991) had followed the fate of 18 water soluble azo dyes in the activated sludge process. They discovered that 11 compounds were found to pass through the activated sludge process substantially untreated, 4 were significantly adsorbed and 3 were apparently biodegraded.

Kanekar and Sarnaik (1991) had established a laboratory-scale activated sludge process to reduce the pollution load of a dye-industry waste containing aniline, phenol, methyl violet and rhodamine B as the major compounds. A microbial sludge

adapted to the waste and used as a bioinoculum. The results showed a significant decrease in the optical density of the colour of the waste from an initial 0.915 to 0.360 at 580 nm. Microorganisms isolated from sludge were identified as *Pseudomonas alcaligenes* and *P. mendocina.*

Idaka *et al.* (1985) had studied specially the removal of congo red, orange II and crystal violet by acclimated activated sludge. Data revealed the improvement of removal capability of the activated sludge by repeated culture.

Pagga and Brown (1986) had tested 87 dyestuffs in short-term aerobic biodegradation tests. In their study, typical commercial products were used instead of relatively simple dyestuff model compounds as in many other studies. There was substantial colour removal for the majority of the dyestuffs tested. Of them, seven dyestuffs indicated a simultaneous drop in both decolorization and DOC (Dissolved Organic Carbon). Thus, they were classified as the "possibly biodegradable" category.

Urushigawa and Yonezawa (1977) began an investigation very early to deal with the relationship between the biodegradation of azo compounds by activated sludge and their molecular structure. The experiment came up with the discovery that hydrogen-, chloro-, methyoxyl- and nitro derivatives other than methyl derivatives were difficult to be decolorized, but only amino and hydroxyl derivatives were decolorized.

Besides the aerated activated sludge processes, the anaerobic treatment

received equal attention. Manulyak *et al.* (1987) had suggested the use of anaerobic bioreactor which contained immobilized anaerobic microorganisms. Time of retention of the waste water in the bioreactor was 48 hours. Destruction of the dyes, which was indicated spectrophotometrically, could reach 87%.

Yang (1990) had developed the Anaerobic-Aerobic-Biological Carbon Treatment Process which showed high operative efficiency. This process had been put in the pilot scale test in several dyeing mills for several years. The quality and quantity of the wastewater was first regulated in the regulating tank. Then the anaerobic tank was used to change the organic substance with long chains into chemical substances with short chains which were degraded by microbes in a contact oxidation tank. Some were assimilated as nutrient by the microorganisms. Finally, the remaining substances were removed by the joint reaction of microbes and activated carbon in the bio-carbon tower. The dyeing wastewater after this treatment had a significant drop in BOD_5 from 120-190 mg/l to 2-5 mg/l as well as a 10 fold decrease in colour.

In order to optimise the efficiency of the activated sludge processes, the biodegradability characteristic of the process has been studied. Such Studies are important to evaluate and compare the biodegradability potential of facilities discharging similar waste effluents (Brown and Weintraub, 1982).

B. Degradation by pure culture

With regard to the studies of degradation by pure culture, azo dyes are the

class that are the most intensively investigated. It is because azo dyes share a large portion of the dyestuffs market and are popularly used in different fields. Therefore, the azo dyes will be used as typical example to illustrate the studies of dyes biodegradation in the following discussion. Pure culture study began as early as 1937 when decolorization of azo dyes was found in spoiled dairy products. Since then, the investigation of azo dyes degradation has been active.

1. Degradation of azo dyes by pure culture

Degradation of azo dyes has been demonstrated in various source including intestinal microflora, liver enzymes and bacteria from environment. Chung *et al.* (1978) had carried out a study of reduction of seven azo dyes by cell suspension of predominant intestinal anaerobes. The activity was optimal at pH7.4 in 0.4 M phosphate buffer but inhibited by glucose. In one of the strains, *Bacteroides thetaiotaomicros*, flavin mononucleotide could cause a significant enhancement of azo reduction. Brown (1981) tried to evaluate the data on the reduction of high-molecular-weight polymeric azo and nitro dyes by intestinal bacteria and their enzymatic extracts. He found that the oxygen sensitive reduction of such dyes appeared to be mediated by low-molecular-weight electron carriers.

Many other microorganisms have been isolated from the polluted environment by dyestuffs. *Bacillus subtilis* (IFO322), a bacterial inhabitant in the activated sludge, was found to be an azo-assimilating bacterium (Ogawa *et al.*, 1988). Idaka *et al.* (1978) had isolated *Aeromonas hydrophila* var. 24B from the draining ditches of dyestuff factories. This strain was able to degrade a number of azo compounds but

the growth was inhibited by adding dyes. Aniline and p-phenylenediamine were obtained as the products of reduction cleavage of aminoazobenzene. So (1989) had screened the strict aerobe *Acetobacter liquefaciens* which could degrade methyl red into anthranilic acid and N, N'-dimethyl-p-phenylene.

The group of Ogawa had isolated some microbial strains in the sludge from the sewage discharged form azo dye factory (Idaka *et al.*, 1987b). These strains were able to assimilate the azo dyes. Among them, strain 13NA was identified as *Pseudomonas cepacia*. It was then named as *Pseudomonas cepacia* 13NA. Its reductive metabolism of aminoazobenzenes and the oxidative pathway that followed had been thoroughly studied (Idaka *et al.*, 1987b; Idaka *et al.*, 1987c)

Kulla (1981) and Kulla *et al.* (1984) had tried to alter the substrate specificity of the azo-dyes assimilating strains. They had firstly isolated a microorganism form soil that grew with a simple azo compound (4,4'-dicarboxyazobenzene, DCAB) as sole carbon, nitrogen and energy source. This strain was tentatively identified as *Flavobacterium* sp. It degraded DCAB into 4-aminobenzoate. Kulla tried to develop the degradative ability towards more complicated azo dyes of Orange I and Orange II types by means of continuous culture. Growth in the chemostat was limited by DCAB while carboxylated orange I and carboxylated orange II were added in excess. After hundreds of generations, strains which grew with carboxylated Orange I and II as sole carbon, nitrogen and energy source were developed. The evolved strains were highly substrate specific.

In further experiment, the corresponding sulfonated dyes of Orange I and

Orange II were added in excess in the chemostat. However, only cometabolism was observed. The degradative ability of carboxylated Orange II seemed to be located on the plasmid since, when carboxylated Orange II-positive stains were grown for 10 generations in nutrient broth at 40°C, more than 99% of the progeny had lost the degradative ability irreversibly.

Although the majority of azo-dyes degrading microorganisms isolated are bacteria, there are also reports about fungal degradation of azo dyes. Cripps *et al.* (1990) had demonstrated the aerobic biodegradation of three azo dyes: Tropaeolin O, Congo Red and Orange II by the white rot fungus, *Phanerochaete chrysosporium.* The decolorization of three dyes was the most extensive in ligninolytic cultures. Nevertheless, substantial decolorization could also be observed in nonligninolytic cultures. The mechanism of decolorization was closely related to the lignin-degrading system of the white rot fungus. Incubation with crude lignin peroxidase resulted in decolorization of Orange II and Tropaeolin O but not Congo Red. This indicated that other enzymes system other than lignin peroxidase may be involved in the first step of the degradation of azo dyes.

2. Metabolic pathway of azo dyes biodegradation

As early as 1911, sulphanilic acid had been found in the urine of dogs fed with Orange I. Thus, it was firstly suggested that the initial step of degradation of azo dyes involved the reductive cleavage of the azo group. Later, azo reductase activity was demonstrated to active in the liver as well as the microflora of the gastro-intestinal tract. For food dyestuffs, as they are generally water-soluble, sulphonated

acid dyestuffs, there is little adsorption along the gastro-intestinal tract. Therefore, the gut microflora were believed to be more important than the liver reductase system in metabolizing the azo dyes.

Idaka *et al.* (1987b) had described the reductive and acetylating pathways of aminobenzenes for *Pseudomonas cepacia* 13NA. The metabolism of aminoazobenzenes by resting cells of *P. cepacia* 13NA was supposed to cause reductive cleavage of the azo bond and then the acetylating amine. Part of the aminoazobenzenes was also acetylated and the acetamidoazobenzenes so formed was subjected to reductive cleavage in the same manner of aminobenzene. For further studies, the oxidative pathway was discovered after the reductive cleavage of the azo bond (Idaka *et al.*, 1987c). The metabolism identified included o-aminophenol, m- and p-acetamidophenol and 3,4-dihydroxyacetanilide.

Idaka *et al.* (1978) tried to extract and identify the degradation metabolites of p-aminoazobenzene by *Aeromonas hydrophila* var. 24B. Aniline and p-phenylenediamine were obtained as the products of reduction cleavage of p-aminoazobenzene. The yield of p-phenylenediamine was relatively low compared with that of aniline. This was possible due to the further metabolism of p-phenylenediamine.

Kulla (1981) had demonstrated the degradative pathway of 4,4'-dicarboxyazobenezene by *Flavobacterium* sp. (later confirmed to be *Pseudomonas* sp.). The degradation products were 4-aminobenzoate.

The reductive cleavage of the azo bond is characteristic of the metabolism of azo dyestuffs. The azo linkage is reduced to form aromatic amines which can be toxic and carcinogenic. Biodegradation of azo dyes can occur in both aerobic and anaerobic system. However, the consequences are not the same. Under anaerobic condition, the decolorization is rather easy and it is elicited by a number of microorganisms through non-specific enzymes. The process usually come to a metabolic standstill and the accumulation of aromatic amines would be resulted. On the other hand, degradation under aerobic condition is accomplished by enzymes with high substrate specificity and the process can lead to complete metabolism of the products.

3. Enzymology of azo dyes metabolism

With regard to the enzymology of azo dyes biodegradation, the enzyme, azoreductase, has been confirmed to be responsible for the initial reductive cleavage of the azo bond. A highly purified cytosolic azoreductase isolated from the livers of 3-methylcholanthrene-treated rats had been characterized by Huang *et al.* (1979). Anaerobic titration of the enzyme with NADPH as the electron donor indicated that one mole of enzyme can accept 4 reducing eq, or 2 electrons/mole of enzyme-bound FAD. This enzyme catalysed the reduction of methyl red under anaerobic conditions. Two moles of NADPH were required for the reduction of 1 mole of methyl red. Dicumarol strongly inhibited the azo reductase-catalysed reduction of methyl red. It inhibited the reaction by interfering with electron transfer form NADPH to enzyme-bound FAD. The purified azoreductase from rat liver seemed to be identical with rat DT-diaphorase.

Zimmermann *et al.* (1982) had reported the successful purification of Orange II azoreductase from *Pseudomonas* species strain KF46. This azoreductase was induced 80-fold by both Orange II and carboxyl-Orange II. Through two successive runs of affinity chromatography using two chromatographic media with different triazinyl dyes as ligands, the enzyme was purified 120-fold with 43% yield. The purified enzyme was a monomer with a molecular weight of 30,000. Its Km values were 1.5μM for NADH. Studies on the substrate specificity revealed the following features:

(a) a hydroxyl group in the 2-position of the naphthol ring is required;

(b) Charged groups in proximity to the azo-groups and a second polar substituent on the dye molecule hinder the reaction;

(c) electron withdrawing groups on the phenyl ring enhance the reaction rate.

Later, Zimmermann *et al.* (1984) reported the purification of another closely related azoreductase, Orange I azoreductase of *Pseudomonas* strain K24. It was purified 80-fold with 17% yield to electrophoretic homogeneity. When compared with the Orange II azoreductase, they shared some common properties including monomeric structure, their specificity for NADPH and NADH as cofactors, the range of their Km values for substrates and cofactors as well as reactivity towards a series of substrate analogs. However, they differed in molecular weight (21,000 and 30,000) and the position of hydroxyl group on the naphthol ring. Although these two enzymes did not exhibit immunological cross-reaction with each other, material which cross-reacted (CRM) with both anti-Orange I and anti-Orange II azoreductase serum had been found. The CRM may be a precursor protein of these two azoreductase.

Idaka *et al.* (1987a) purified an azoreductase produce by *Pseudomonas cepacia* 13NA. This enzyme showed an optimum temperature of 37°C and an optimum pH of 6.8-7.0. The degradation required NADPH or NADH as cofactors but NADPH was more effective.

4. Biodegradation of other dyes

Compared with azo dyestuffs, these are relatively few literature concerning the biodegradation of dyes in other classes. Glenn and Gold (1983) examined the polymeric dyes Poly B-411, Poly R-481 and Poly Y-606 in lignin biodegradation assays. The decolorization of these dyes by the white rot basidiomycete. *Phanerochaete chrysosporium* occurred during secondary metabolism and was suppressed in the presence of a high nitrogen level. The process was strongly dependent on the oxygen supply. Inhibitors of lignin degradation also inhibited dyes decolorization. Pleiotropic mutant lacking ligninolytic activity was not able to decolorize the polymeric dyes. All these results suggested that the ligninolytic degradation activity was responsible for the decolorization.

The white rot fungus , *P. chrysosporium* was subjected to test for degradative ability of other dyes classes. Bumpus and Brock (1988) tested the biodegradation of crystal violet by this fungus. Identification of the metabolites indicated the sequential N-demethylation of the parent compound. Decolorization of crystal violet proceeded in both ligninolytic and nonligninolytic cultures suggesting that in addition to the lignin-degrading system, another degradation mechanism of crystal violet existed in this fungus. Besides crystal violet, six other triphenylmethane dyes could be also

degraded by the lignin-degrading system of *P. chrysosporium*.

Cripps *et al.* (1990) demonstrated the degradation of the heterocyclic dye, azure B by *P. chrysosporium*. The decolorization was the most extensive in ligninolytic cultures but substantial decolorization also occurred in nonligninolytic cultures.

Biodegradation of crystal violet was also found in oxidative red yeast (Kwasniewska, 1985). After 4 days incubation, no trace of crystal violet could be observed in the cultures of *Rhodotorulae* sp. and *Rhodotorulae rubra*.

For the xanthene dyestuffs, monohalogenated fluoresceins were reported to be degraded to fluorescein in rats. Rhodamine B was converted to 3',6'-disminofluoran through stepwise de-ethylation in the liver cell microsomes (Clarke and Anliker, 1980).

VI. General properties of indigoid dyes

Indigoid dyes are important historically. Indigo itself is one of the oldest known dyes. The earliest records of use of indigo could be traced back to the valleys of the Tigris, the Euphrates, the Indus and the Nile. Indigo is a kind of natural dye. A large number of plants are for the production of indigo. Those in the genus *Indigofera* are the most valuable (Lynn, 1985; Trotman, 1970). Nowadays, indigo is the only natural dye which retains the same importance as a textile dye although its production has been taken over by chemical synthesis.

After the synthesis and commercial production of artificial alizarin in 1889, it was obvious that the synthesis of indigo would be an immediate target for dye chemists. By 1870, the first small quantity of indigo was produced in the laboratory of two German chemists, Engler and Emmering. The chemical structure of indigo was later worked out by Baeyer in 1883. Numerous processes have been developed for the artificial synthesis of indigo but that of Haumannf and Pfleger is one of the widely used processes. Aniline is reacted with chloracetic acid to give phenylglycine and ring closure is effected using metallic sodium and ammonia to form indoxyl which by condensation and oxidation gives indigo (Fox and Pierce, 1990).

Indigo imparts an attractive blue colour and natural look to the fabric. The resulting dyed material retains a rich, pleasant and beautiful blue colour to the end of the life of even the most vigorous used garment. It is doubtless why indigo dyed jeans and denims are still popular and held in high esteem by todays's young generation.

The general properties of indigo are summarised as follows:

(a) Solid is a dark blue powder possessing a copper-red iridescence.

(b) Insoluble in water, alcohol, ether and dilute acid

(c) Low solubility in most organic solvents such chloroform, aniline.

(d) Can be crystallized from chloroform, nitrobenzene, aniline and certain other solvents.

(e) In a sealed tube, it melt at 390-392°C to a purple-red liquid which decompose rapidly. The sublime vapour is fiery red with a violet tinge.

(f) Combined with mineral acid to give salt, but easily decomposed by water.

When the dye is suspended in acetic acid, benzene or chloroform and react with dry HCl gas, the salt is formed and dissolved readily in these solvents.

(g) React with strong alkaline and produce a green powder.

(h) Strong oxidation agents such as nitric acid or chromic acid can separate the two indole fragments with the formation of isatin.

(i) reduction by ferrous sulphate and alkaline produce indigo white. Somewhat soluble in boiling water, alcohol and ether with yellow colour.

(j) alkyl and acyl derivatives can also be produced.

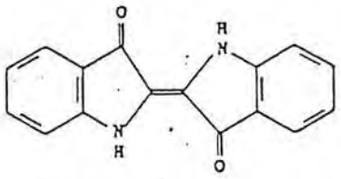
(k) Under ozonation, indigo react to form isatin and isatoic anhydride (Grosjean *et al.*, 1988).

(l) The benzene ring can be substituted by sulfuric acid, halogen and nitro derivatives. Preferences for entry in position 5 > 7 > 4. Many other dyes have been derived from these derivatives. These derivatives are more water soluble.

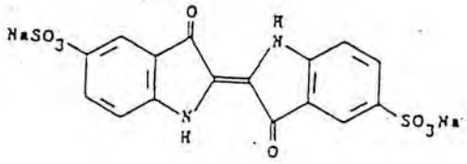
One of these is the indigo-5,5'disulfuric acid (indigo carmine). This is highly soluble in water (1 gm per 100 ml water) and is blue in colour. Thus, it is suitable for use as testing agent for the study of microbial degradation of indigoid compounds.

Indigo carmine is used as dye, in a functional kidney test, in colouring nylon surgical sutures. It is also used as reagent for the detection of nitrate, chlorate and in testing milk. It is an approved dye for use in food and ingested drugs. However, it was showed to be mutagenic in some test system (Table 1).

VII. Biosynthesis of indigo



A



B

Figure 2: Chemical structure of indigo (A) and indigo carmine (B).

Table 1 : Table showing the mutagenic, carcinogen and clastogenic effects of indigo carmine in different test system. (modified from Roychoudhury and Giri, 1989)

TEST SYSTEM	POSITIVE EFFECT	NEGATIVE EFFECT
Salmonella typhimurium		-
Saccharomyces cerevisiae		-
Rat embryo cells	+	
Bone marrow cells of mice	+	
CHO cells in vitro	+	
Human lymphocytes		-
Allium cepa	+	

Indigo is originally extracted from plants. However, the production of indigo from microorganisms was firstly reported in 1928. A strain of *Pseudomonas indoloxidans* was found to produce indigo from indole. Subsequent study (Oshima *et al.*, 1965) revealed that indoxyl was an intermediate of the reaction and the hydroxylation of indole to indoxyl was catalysed by an oxygenase.

Sebek and Jäger (1962) reported another strain of *Chromobacterium violaceum* which also produce indigo under particular conditions. This strain normally produced violacein from indole. However, rapid lyophilization of washed cells would inactivates enzymes of the violacein pathway and indole was metabolized to indigo. Indoxyl was a likely intermediate in the reaction.

With the rapid development of genetic engineering, DNA fragment encoding the synthesis of indigo have been cloned. Ensley *et al.* (1983) had cloned a fragment of plasmid NAH7 from *Pseudomonas putida* PpG7 and expressed in *Escherichia coli*. Indigo was formed in the cultural medium. The formation of indigo is enhanced in the presence of tryptophan or indole. It was suggested that indigo formation was due to the combined activities of tryptophanase and naphthalene dioxygenase (Figure 3). The naphthalene dioxygenase coding genes was then cloned by Kurkela *et al.* (1988) based on their ability of converting indole to indigo. The coding region for this enzyme activity was sequenced and three successive open reading frames were found. The corresponding gene products were identified and all were necessary for the enzyme activity.

Keil *et al.* (1987) had cloned the entire operon coding for the enzymes

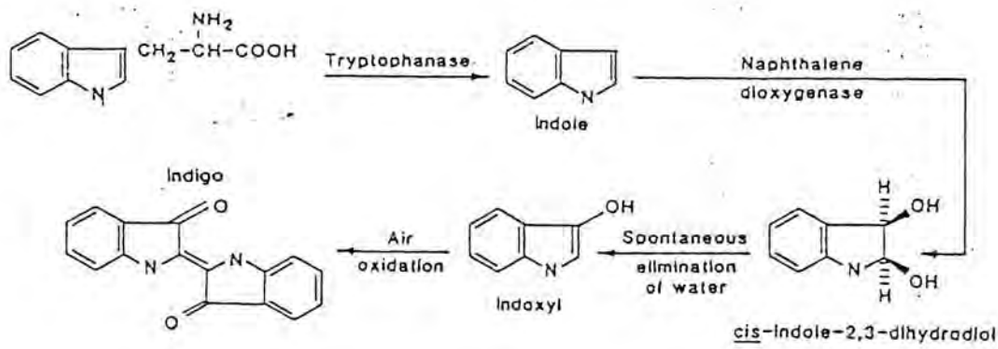


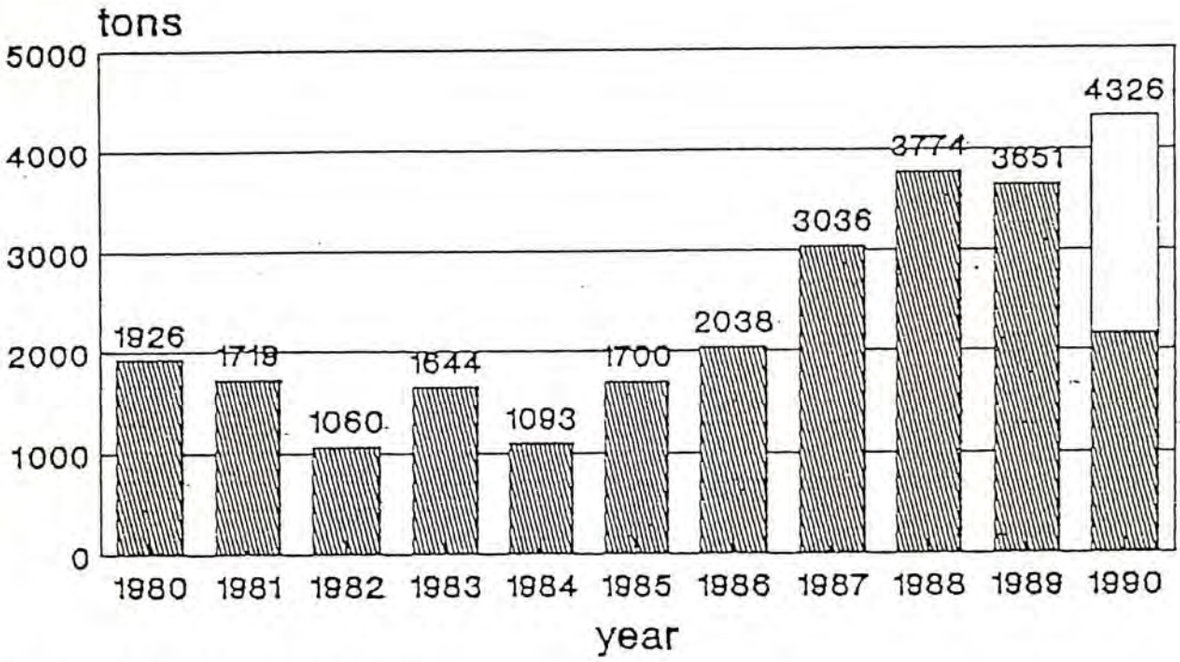
Figure 3: Proposed pathway for indigo biosynthesis in a recombinant strain of *E. coli*. Indole is formed from tryptophan by tryptophanase, a natural enzyme in *E. coli*. Naphthalene dioxygenase formed by expression of the cloned *Pseudomonas* DNA oxidizes indole to indigo. cis-2,3-Dihydroxy-2,3-dihydroindole and indoxyl have not been isolated. Their inclusion is based on the known activities of aromatic hydrocarbon dioxygenases and established mechanisms for the chemical synthesis of indigo. (Ensley *et al.*, 1983)

responsible for conversion of toluene to benzoates from TOL plasmid pWW53. A central region of 2.9 kbp complemented an *xylA* (xylene oxygenase) mutant of *P. putida* mt-2 and was capable of converting indole to indigo when transformed into *E. coli* and *P. putida*. This reaction was due to monooxygenase. It was proposed that the region encoded xylene oxygenase activity capable of direct monohydroxylation of indole to 3-hydroxyindole which then spontaneously dimerized to form indigo.

Mermod *et al.* (1986) found that *E. coli* cells containing a cloned fragment of *P. putida* TOL plasmid pWW0 produced indigo. The *xylA* gene was reponsible for this phenotype. Indole was shown to be a precursor in the reaction. Wu *et al.* (1989) used *Pseudomonas* sp. S13 harbouring naphthalene degradation plasmid as donor and *E. coli* as recipient. Conjugants and transformants with the plasmid were obtained. They were able to synthesize indigo in the medium. The production of indigo was increased in the presence of tryptone or indole. Indigo formation was enhanced if the bacteria was grown in a medium supplemented with either 0.1% of naphthalene or 1% of salicylic acid.

VIII. Indigo as dye in Hong Kong

Textile industry is one of the major industries in Hong Kong and denim products has a big share of the market. Therefore, there is an abundant quantity of indigo imported into Hong Kong for textile use (Figure 4). During the manufacture process, considerable amount of indigo escapes into the environment. Sewage from the dyeing factories contains a high level of different dyestuffs. This situation is easily observed particularly in the industrial zones concentrating with dyeing factories such



* The figure of 1990 is expected.
(Jan - July, 2163 tons)

Figure 4: Annual imports of indigo to Hong Kong

as Tsuen Wan and Kwai Chung.

In 1988, there is altogether 70 tonnes dyestuffs wastes. This huge amount of waste undoubtedly causes an environmental problem and should receive much attention. In recent years, the Environmental Protection Department (EPD) considered much more seriously about the measures of counteracting the pollution problems in Hong Kong. In 1989, EPD has published the 'Draft Waste Disposal Plan for Hong Kong'(Environmental Protection Department, 1989). In this report, land-fill and incineration are still two major waste disposal methods for Hong Kong. However, their disadvantages have been thoroughly evaluated. New alternative methods are urged to develop for effective waste treatment.

IX. Purpose of study

The majority of the studies in the biodegradation of dyestuffs has been focused on the azo dyes while other dyes classes especially the insoluble dyes such as indigo and sulphur black are neglected. However, the production of these dyes is also abundant. As in Hong Kong, indigo is an important dye used in textile industry and at the same time, an environment problem has been caused by the disposal of untreated dyeing wastewater. Facing the challenge of exploring a new ground in biological research as well as meeting the need of the society, this project is launched.

Since there is extensive dumping of indigo into the environment during the past years, there should be microorganisms evolved to degrade indigo. The present research is based on this rationale and by using indigo carmine as an model compound of the indigoid dyes, try to achieve the following goals:

I. Isolate indigo degrading strains from the polluted environment and then identify them. This step will be a breakthrough since for the past 20 years, no literature had reported any microbial degradation of indigoid dyes (Figure 5).

II. Optimise the growth and decolorization ability of the isolated degrading strains.

III. Extraction and identification of the degradation products so as to establish the metabolic pathway of indigoid dyes by these strains.

```
File    5:BIOSIS PREVIEWS 69-89/FEB BA8703;RRM3603
          (C.BIOSIS 1988)

       Set   Items   Description
       ---   -----   -----------
?ss indigo
        S1      290   INDIGO
?ss degrading or degradation or decompose or decomposition or decolor
        S2     3372   DEGRADING
        S3    42545   DEGRADATION
        S4      532   DECOMPOSE
        S5     9608   DECOMPOSITION
        S6       32   DECOLOR
        S7    54032   DEGRADING OR DEGRADATION OR DECOMPOSE OR DECOMPOSITION
                      DECOLOR
?ss microorganism or bacteria or fungi
        S8     5238   MICROORGANISM
        S9    83294   BACTERIA
       S10    28881   FUNGI
       S11   109621   MICROORGANISM OR BACTERIA OR FUNGI
?ss s1 and s7 and s11
              290   S1
            54032   S7
           109621   S11
      S12        1   S1 AND S7 AND S11
?type s12/3/1

 12/3/1
0018754168      BIOSIS Number: 86125954
  ENZYMATIC DEGRADATION OF URINARY INDOXYL SULFATE BY PROIDENCIA-STUARTI
AND KLEBSIELLA-PNEUMONIAE CAUSES THE PURPLE URINE BAG SYNDROME
  DEALLER S F; HAWKEY P M; MILLAR M R
  DEP. MED. MICROBIOL., LEEDS GEN. INFIRMARY, LEEDS, ENGLAND LS2 9JT.
  J CLIN MICROBIOL   26 (10). 1988.  2152-2156.   CODEN: JCMID
  Language: ENGLISH
```

Figure 5: Record of on-line literature search for indigo degrading microorganisms. Not a single reference has been found for the past 20 years (69-89).

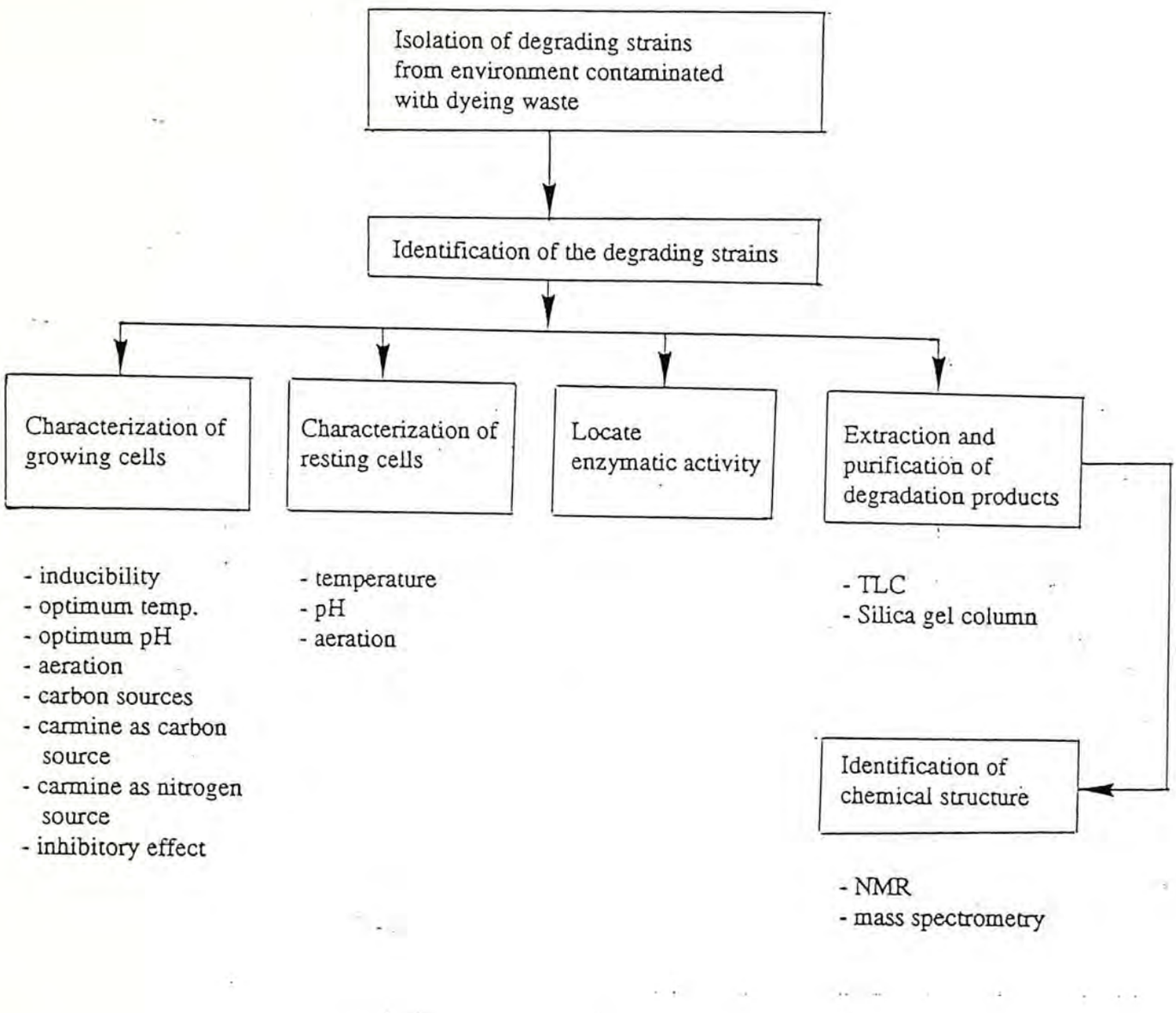


Figure 6: The general outline of this project.

MATERIALS AND METHODS

I. Preliminary studies on the stability of indigo carmine

A. Chemical tests:

To 2 ml of 25 ppm indigo carmine solution , 0.1 ml of the following reagents was separately added. Reactions were allowed to proceed for 1 hour and then the absorbance of the reaction mixture were measured at 609nm :

1. Acid

   --- strong hydrochloric acid (10N)

2. Reducing agent

   --- iron powder in dilute hydrochloric acid (1N)

3. Oxidizing agent

   --- perchloric acid (70%)

B. Heat stability test

Indigo carmine solution was autoclaved at 121°C, 16 lb/in^2 for 30 minutes. Both the solutions before and after treatment were scanned from 200-700 nm by using Beckman Du-7 spectrophotometer. The two profiles were compared to look for any differences.

C. Light sensitivity test

A flask containing 50 ppm indigo carmine in distilled water was left on the bench. The flask was illuminated by natural dark and light cycle. After 48 hours, the absorbance of the solution was measured at 609nm to detect any change in the intensity of the colour.

II. Standard curve of indigo carmine

To prepare serial dilutions of indigo carmine solution from 50 ppm. The absorbance of the dilutions at 609nm were measured. The absorbance was then plotted against the concentration to construct a standard curve for indigo carmine.

III. Isolation of indigo carmine degrading bacteria

Soil samples were collected at two industrial sites in which dyeing factories were concentrated and the surrounding environment was severely polluted by dyestuffs. These sites included Ho Chung and Tsuen Wan. The collected soil samples were first suspended in water. Serial dilutions were prepared and then plated on the Isolation Medium (I.M.).

Composition of the I.M. : tryptone (Difco) 10 g/l, yeast extract (Difco) 1 g/l, ammonium chloride (Merck) 1 g/l and indigo carmine (Sigma) 0.5 g/l.

The plates were incubated at 37°C for the colonies to grow up. Indigo degrading strains were able to form clear halo around the colonies. These strains were restreaked on I.M. plates containing indigo carmine to purify the strains and to confirm the halo forming ability of theses strains.

IV. Identification of isolated strains

The isolated bacterial strains were identified by fatty acid profile comparing method which was performed by Microcheck, Inc. Each isolate was subcultured on trypticase soy broth agar (TSBA) to produce enough cells for whole cell fatty acids. The bacterial identification system consisted of a gas chromatograph with a flame ionization detector, an autosampler, an integrator and a computer. The identification system used a five percent methyl phenyl silicone capillary column. The bacterial whole cell fatty acid extracts was injected into the system. The computer named the fatty acids and compared the fatty acid profile of the unknown bacteria to the profiles of the 7000 strains in the database through the use of software which used covariance matrix, principal component analysis, and pattern recognition.

V. Characterization of batch culture of *Micrococcus* sp. H-12

A. Inducibility of decolorization ability

Two separate I.M. batch culture were prepared in which one contained 50 ppm indigo carmine whereas another was free of the dye . 1% inoculum of overnight culture was added to the two cultures respectively. The cultures were incubated at 37°C shaking at 200 r.p.m. for 24 hours. Bacterial cells from these two cultures were centrifuged at 10,444 x g for 15 minutes and then washed with 50 mM phosphate buffer (pH 7.0). Finally, the cells were resuspended in equal volume of 50 mM phosphate buffer.

The decolorization ability of the resting cells was determined according to the following assay conditions :

0.1M Phosphate buffer (pH 7.0)	0.5 ml
500 ppm indigo carmine	0.1 ml
resting cells	1.0 ml

The reaction mixtures was made up to 2 ml with distilled water and incubated at 37°C for 1 hour. At the end of incubation, the cells were spinned down by microfuge for 2 minutes. Amount of indigo carmine left was determined by the absorbance of the supernatant at 609 nm.

B. Growth and decolorization kinetics in indigo carmine

H-12 was grown in I.M. containing indigo carmine in a rotatory shaker (New Brunswick Scientific Co.) at 37°C, 200 r.p.m. for overnight. 1% inoculum was then added to fresh IM medium containing 50 ppm indigo carmine. The same incubation condition was applied as the above.

At certain time intervals, aliquots of culture were taken out. The cells were spinned down by microfuge for 2 minutes. The supernatant was saved for the assay of indigo carmine level. The bacterial cells were washed with 50 mM phosphate buffer (pH 7.0) once and finally resuspended with phosphate buffer of original volume.

Growth was monitored by the absorbance of the resuspended cells

solution at 650 nm. Indigo carmine level was assayed by measuring the absorbance of the supernatant at 609 nm. Decolorization of indigo carmine was determined according to the following calculation :

$$\text{Percentage of decolorization} = \frac{\text{Initial Absorbancy - Absorbancy at Time t}}{\text{Initial Absorbancy}}$$

C. Effect of Temperature on growth and decolorization of the bacterial culture.

1% inoculum of overnight culture of H-12 was added to two flasks containing 50 ppm indigo carmine. The cultures were incubated at 30°C and 37°C shaking at 200 r.p.m. respectively. Growth and decolorization of indigo carmine were monitored by the methods described above. On the other hand, H-12 colony was streaked on I.M. plates and incubated at 55°C. The plates were observed after 48 hours for any growth.

D. Effect of pH on growth and decolorization of the bacterial culture.

H-12 was grown in I.M. containing 50 ppm indigo carmine. The pH of the cultures were adjusted to 6.0, 6.5, 7.0, 7.5 and 8.0 respectively by 0.05M K_2HPO_4/KH_2PO_4 buffer. All the cultures were incubated at 37°C shaking at 200 r.p.m. Growth and decolorization of indigo carmine were

monitored by the methods described above.

E. Effect of aeration on the growth and decolorization of the bacterial culture.

Two flasks containing I.M. with 50 ppm were prepared. One was shaken at 200 r.p.m. whereas another was kept at static condition. One percent inoculum of overnight culture of strain H-12 was added. Both of them were incubated at 37°C. At certain time intervals, aliquots of cultures were taken out. Growth and decolorization were monitored by the methods described above.

F. Decolorization of indigo carmine under anaerobic condition.

Colony of strain H-12 was streaked on I.M. plates containing indigo carmine. The plate was put in anaerobic jar in which anaerobic condition was kept by Oxoid gas generating kit. The plates were incubated at 37°C for 48 hours. Ability to decolorize the dye could be indicated by clear halo around the colonies.

G. Effect of carbon source on the growth and decolorization of the bacterial culture.

Strain H-12 was grown in I.M. containing 50 ppm indigo carmine. One percent of various carbon sources were supplied to the bacterial culture to

replace tryptone in the I.M. The carbon sources being studied included glucose, tryptone, acetate, citrate and soluble starch. The cultures were incubated at 37°C shaking at 200 r.p.m. Growth and decolorization were monitored by the methods described above.

H. Utilization of indigo carmine as carbon source.

In order to determine whether indigo carmine could serve as carbon source for strain H-12, the bacterial cells were grown in I.M. free of tryptone. The composition of the modified I.M. was: ammonium chloride 1.0 g/l and yeast extract 1.0 g/l supplemented with 50 ppm of indigo carmine. A control was set up in which no indigo carmine was added.

The two cultures were incubated at 37°C, shaking at 200 r.p.m.. At certain time intervals, aliquots of cultures were taken out for measuring growth and decolorization.

I. Utilization of indigo carmine as nitrogen source.

In order to determine whether indigo carmine could serve as nitrogen source for strain H-12, the bacterial cells were grown in I.M. with glucose in replace of tryptone. The composition of the modified I.M. was: glucose 1% and yeast extract 1.0 g/l. 50 ppm indigo carmine was added initially and at the 24th hour. A control was set up in which no indigo carmine was added.

The two cultures were incubated at 37°C, shaking at 200 r.p.m.. At certain time intervals, aliquots of cultures were taken out for measuring

growth and decolorization.

J. Inhibitory effect of indigo carmine to the growth and decolorization of the bacterial culture

Strain H-12 was grown in I.M. containing 10, 25, 50, 100, 200 and 500 ppm indigo carmine respectively. The cells were incubated at 37°C shaking at 200 r.p.m. At certain time intervals, aliquots of culture were taken out. Growth and decolorization of indigo carmine were monitored by the methods described above. For the cultures containing indigo carmine greater than 50 ppm, the supernatant was diluted 10 folds before measuring absorbance.

$$\% \text{ inhibition} = \frac{\mu \text{ (free)} - \mu \text{ (dye)}}{\mu \text{ (free)}} \times 100\ \%$$

μ (free) = specific growth rate when the culture was free of dye

μ (dye) = specific growth rate in the presence of dye

VI. Characterization of resting cells of *Micrococcus* sp. H-12

In order to study the effects of temperature, pH and aeration on the decolorization ability of strain H-12 resting cells, the resting cells were prepared as outlined below :

Preparation of resting cells

H-12 was grown in I.M. containing 50 ppm indigo carmine. Culture was incubated at 37°C for 24 hours. The bacterial population was at the stationary phase then. Cells were harvested by centrifugation at 10,444 x g for 15 minutes. Cells were washed once with 50 mM phosphate buffer and finally resuspended in it. These adapted resting cells were ready for the following experiments.

Assay condition

0.1M phosphate buffer (pH 7.0)	0.5 ml
500 ppm indigo carmine	0.1 ml
resting cells	1.0 ml

Reaction mixture was made up to 2 ml with distilled water. The reaction mixture was incubated for 1 hour. Afterwards, The absorbance at 609 nm was measured to determined the decolorization level.

A. Effect of incubation temperature

Resting cells were prepared by the methods described above. The reaction mixtures were incubated at various temperature : 4, 15, 25, 30, 37, 50 and 60°C. After one hour incubation, the absorbance was measured.

B. Effect of incubation pH

Resting cells were prepared by the methods described above. Buffers at appropriate pH were used instead of the phosphate buffer whenever necessary. The reaction mixtures were incubated at various pH : 3, 4, 5, 6, 7, 8, 9 and 10. Buffers that had been used were:

pH 3-5 citrate buffer

pH 6-7 phosphate buffer

pH 8-10 borate buffer

After one hour incubation at 37°C, the absorbance was measured.

C. Effect of aeration

Resting cells were prepared by the methods described above. Two separate reaction mixtures were prepared. One reaction mixture was shaken at 200 r.p.m. whereas another was kept at static condition. After one hour incubation at 37°C, the absorbance was measured.

VII. Decolorization by cell-free extract

In order to locate the origin of the degrading enzymes i.e. exo-enzymes or endo-enzymes, strain H-12 was grown in I.M. containing 50 ppm indigo carmine. Culture was incubated at 37°C. Cells were then harvested. The time for cell harvest was critical. It was the best between 15 and 20 hours. The supernatant was fitered through 0.22μ millipore filter paper to remove any bacterial cells and then saved for later assay.

On the other hand, the cells were washed twice with 50 mM phosphate

buffer and then disrupted by French press. Cell-free extract was obtained after the cell debris was removed by centrifugation at 12,062 x g for one hour.

Both the original culture supernatant and the cell-free extract were assayed for enzymatic activity. The assay system was described below:

0.1 ml phosphate buffer (pH 7.0)	0.5 ml
500 ppm indigo carmine	0.1 ml

One ml supernatant or cell-free extract was added to the reaction mixture and the final volume was made up to 2 ml with distilled water. The mixtures was incubated at 37°C for one hour. Absorbance at 609 nm was measured at the end of incubation.

VIII. Identification of degradation products of indigo carmine

A. Preliminary analysis by spectrophotometric method

Adapted bacterial cells of strain H-12 were prepared by inoculate single colony into I.M. containing indigo carmine. The culture was incubated at 37°C shaking at 200 r.p.m. for 24 hours. One percent overnight culture was then inoculated into fresh I.M. containing 50 ppm indigo carmine. The same incubation condition was applied as above. At certain time intervals, aliquots of cultures were taken out. The aliquots were scanned from 200 nm to 700 nm and their profiles were compared.

B. By thin layer chromatographic method

Strain H-12 was grown in modified I.M. containing : glucose 1%, ammonium chloride 1.0 g/l, yeast extract 0.2 g/l and 50 ppm indigo carmine. The culture was incubated at 37°C shaking at 200 r.p.m. Complete decolorization occurred after 24 hours. Then the cells were spinned down by centrifugation at 8000 r.p.m. for 15 minutes twice. The supernatant was subjected to lyophilization by VirTis Lyophilizer for 48 hours. The residue was extracted by 20 ml methanol for three times. Finally, the degradation products from 10 mg indigo carmine in 60 ml methanol was obtained. The extracted materials were spotted on TLC aluminium sheets precoated with silica gel 60 F_{254} (Merck) and using pure methanol as the mobile phase. Degradation products were checked by observation under both short and long wavelength ultra-violet light.

C. Determination of chemical structures of the degradation products.

In order to determine the chemical structures of the degradation products, they were purified by silica gel column (Figure 7). A mixture of degradation products was prepared by growing strain H-12 in modified I.M. containing indigo carmine and extracted by the methods described above. For each time, 15 ml extracted materials was mixed with about 0.5g silican gel and the methanol was completely removed by the rotatory evaporator. The extracted materials were coated on the silica gel and then loaded in a column of silica gel 60 (230-400 mesh) (Merck) of 35 cm in length and 1.5 cm in diameter. Pure methanol was used as the mobile phase. The flow rate was adjusted to 2.5 ml per 5 minutes. Fractions were collected for every 2.5

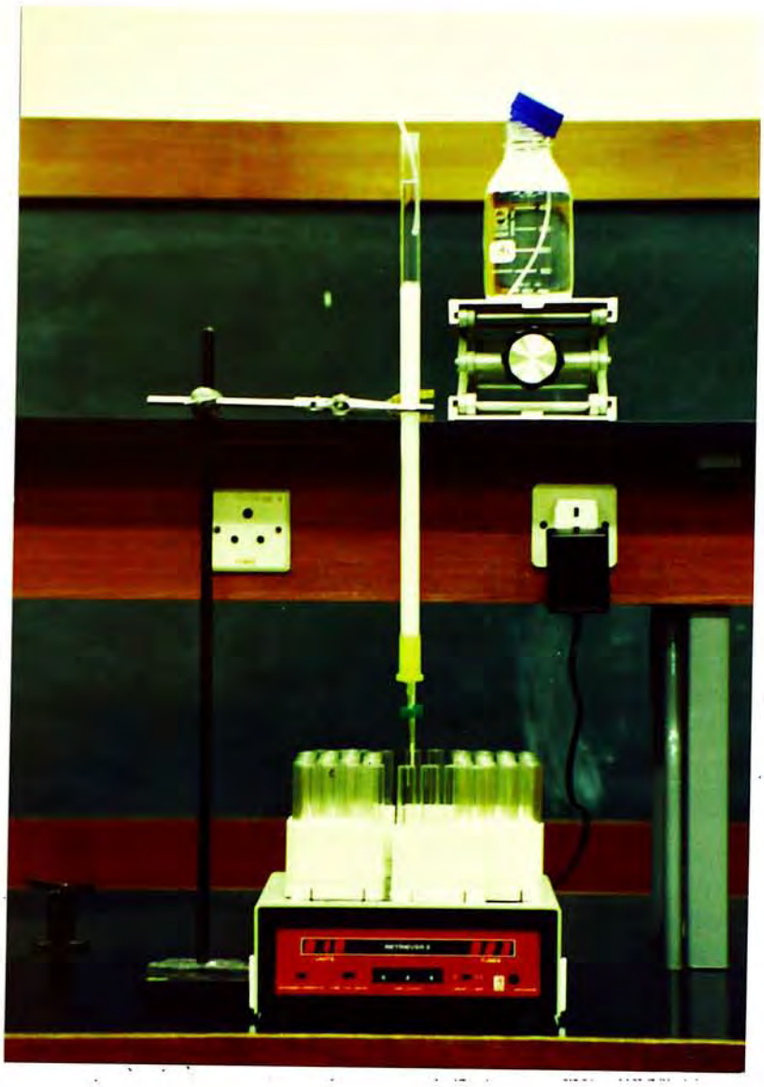


Figure 7: Experimental setup of silica gel column for the purification of the degradation products of indigo carmine.

minutes (1.25 ml). The fractions so collected was checked by UV to locate the components. Purified components were then subjected to chemical analysis of nuclear magnetic resonance (NMR) and mass spectrometry.

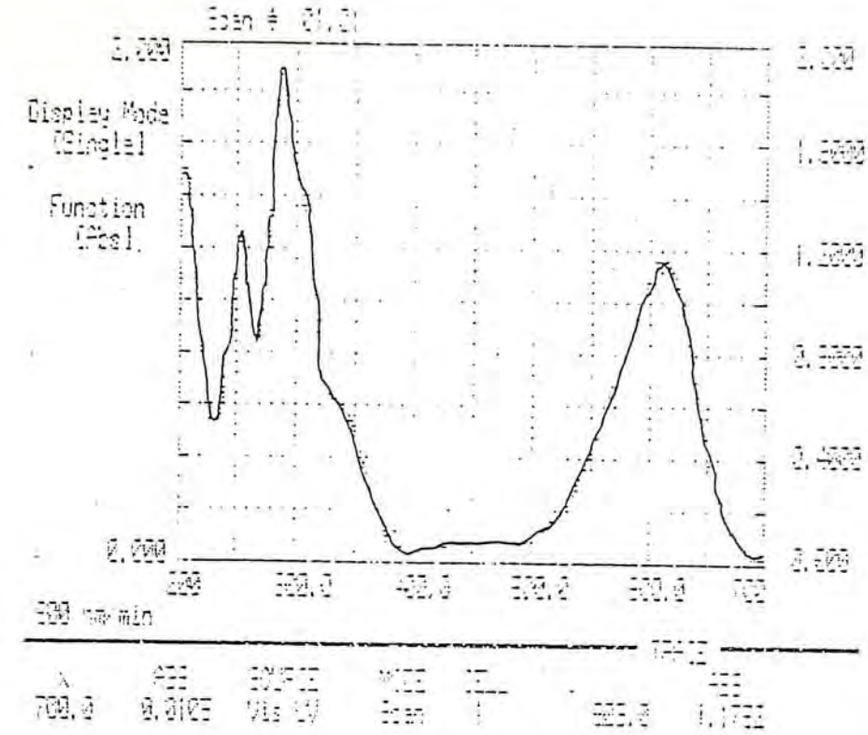
RESULTS

I. Preliminary studies on the stability of indigo carmine

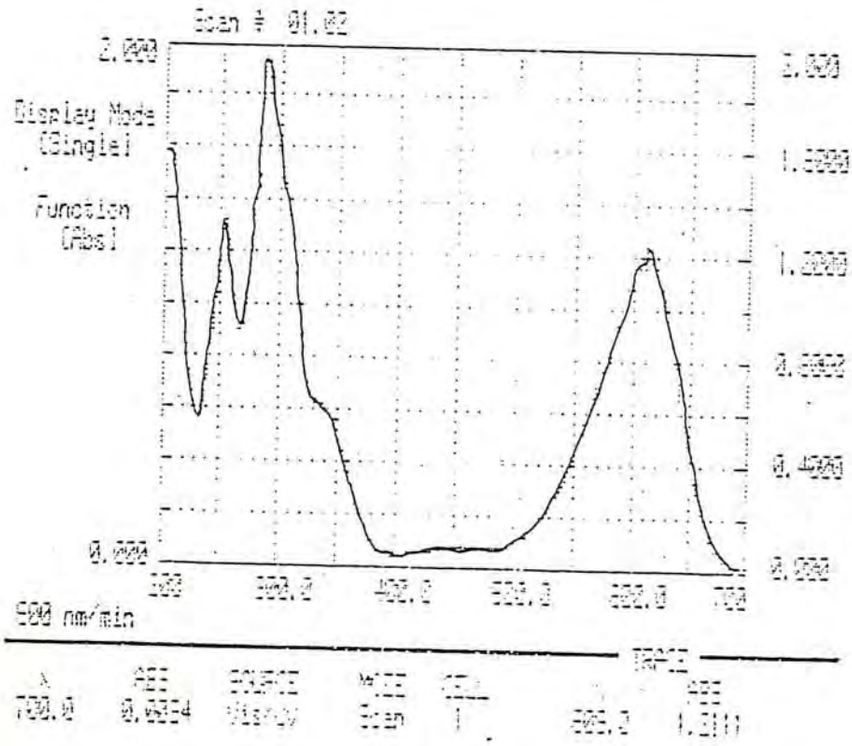
Indigo carmine was stable in all the chemical tests. After one hour incubation with 10N hydrochloric acid, iron powder in dilute hydrochloric acid (1N) and perchloric acid (70%) respectively, there was no observable change in the intensity of the blue colour by naked eye. The final reaction mixtures were further subjected to the measurement of absorbance at 609 nm. The results did not indicate a significant change.

For the heat stability test, indigo carmine was found to be very thermostable. Three peaks appeared in the scanning profile of the aqueous solution of indigo carmine, they were at about 200, 280 and 609 nm (Figure 8). The peak 609nm would be routinely used for the measurement of the concentration of indigo carmine in later experiments. When the scanning profiles of 200-700 nm before and after the treatment were compared, it was found that this dye remained unchanged in terms of the number, positions and the magnitudes of the peaks. The two patterns were essentially the same. Therefore, indigo carmine was sterilized by autoclave when necessary in subsequent experiments.

The solution of indigo carmine was left on the bench and exposed to light for 48 hours. The absorbance after 48 hours remained the same as the original absorbance. So the literature description of light sensitivity of indigo carmine is not a serious problem in our studies.



A



B

Figure 8: Scanning profiles of indigo carmine solution from 200 to 700 nm before (A) and after (B) autoclave. No significant difference can be found between these two curves.

II. Standard curve of indigo carmine

The standard curve was constructed from 0-50 ppm corresponding to absorbance of 0-2.0 at 609 nm (Figure 9). Within this range, the concentration and the absorbance at 609 nm obtained a nearly perfect linear relationship. Thus, the degree of decrease in the absorbance at 609 nm could directly reflect the extent of drop in the dye concentration. Furthermore, this curve was referred in the calculations of indigo carmine content when necessary.

III. Isolation and identification of indigo carmine degrading strains

From the soil samples collected from the polluted environments of Ho Chung and Tuen Wan. Ninety-one strains had been isolated, 40 strains from the waste water of Pollux Dyeing Factory , Ho Chung and 51 strains from the China Textile plant, Tsuen Wan. Most of them are bacteria except three are fungi. The isolated strains were able to form halo on the I.M. plate supplemented with 0.5% indigo carmine. These strains were also effective in decolorizing indigo carmine in liquid medium.

The decolorization ability of these strains were further tested by comparing the size of the halo formed and the rate of decolorization in the liquid medium. Finally, six bacteria strains with the highest performance in the decolorization of indigo carmine were chosen for identification.

The identification was done by the method of comparing the fatty acids profile

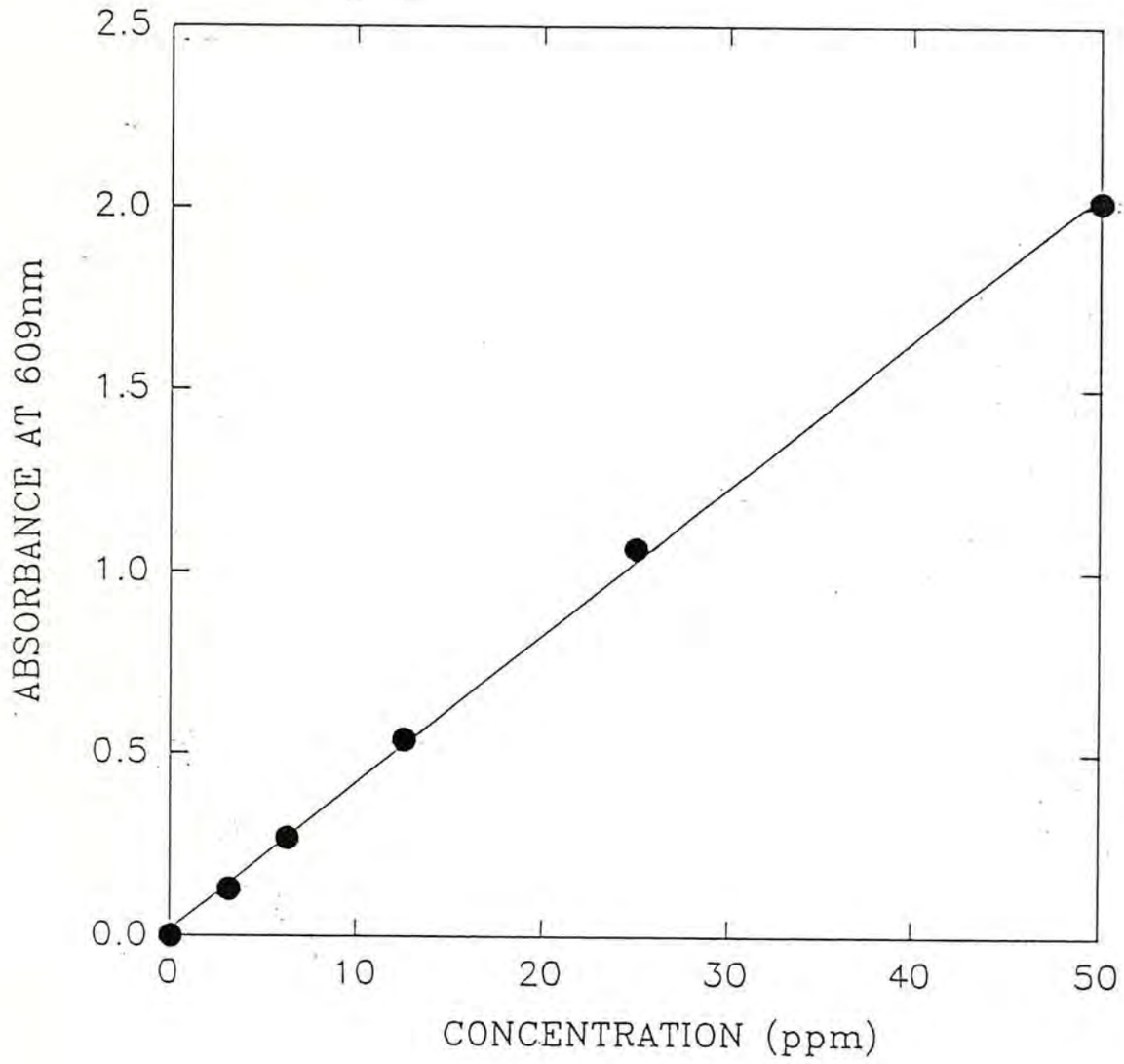


Figure 9: Standard curve of indigo carmine.

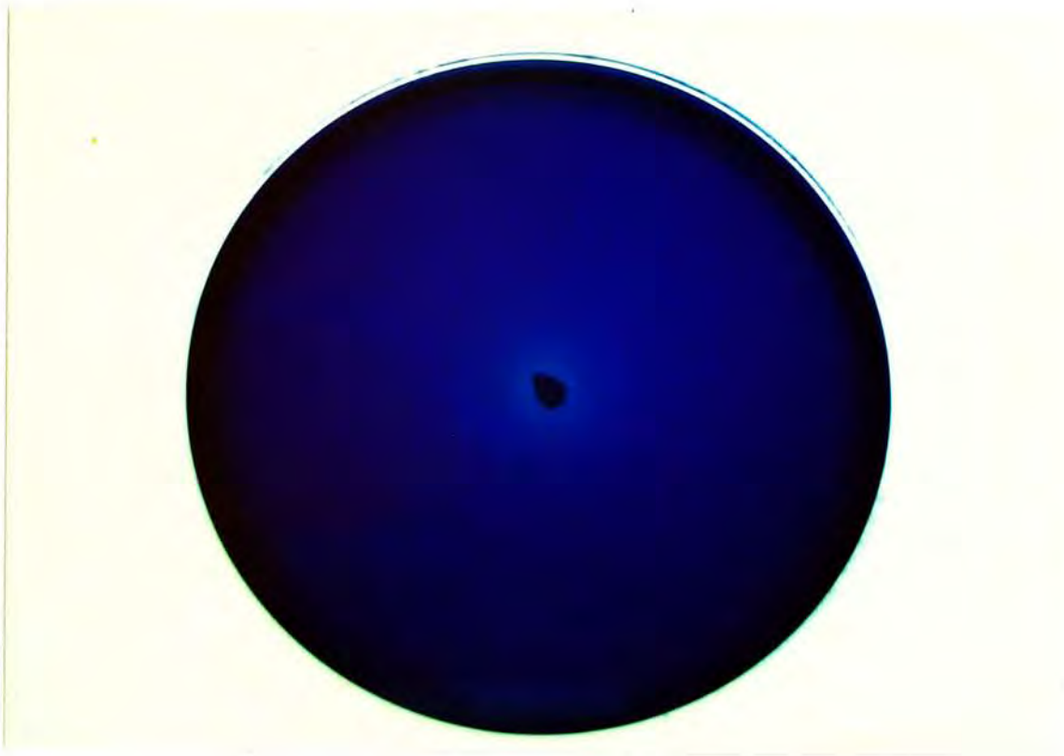


Figure 10: Halo formation by strain H-12 on I.M. plate supplemented with 0.5 g/l indigo carmine.

Figure 11: Decolorization of indigo carmine by strain H-12 in liquid medium :

(A) Initial appearance of I.M. supplemented with 50 ppm indigo carmine.

(B) Culture after 24 hours incubation with cells of strain H-12 removed.

(C) I.M. without treatment for comparison.

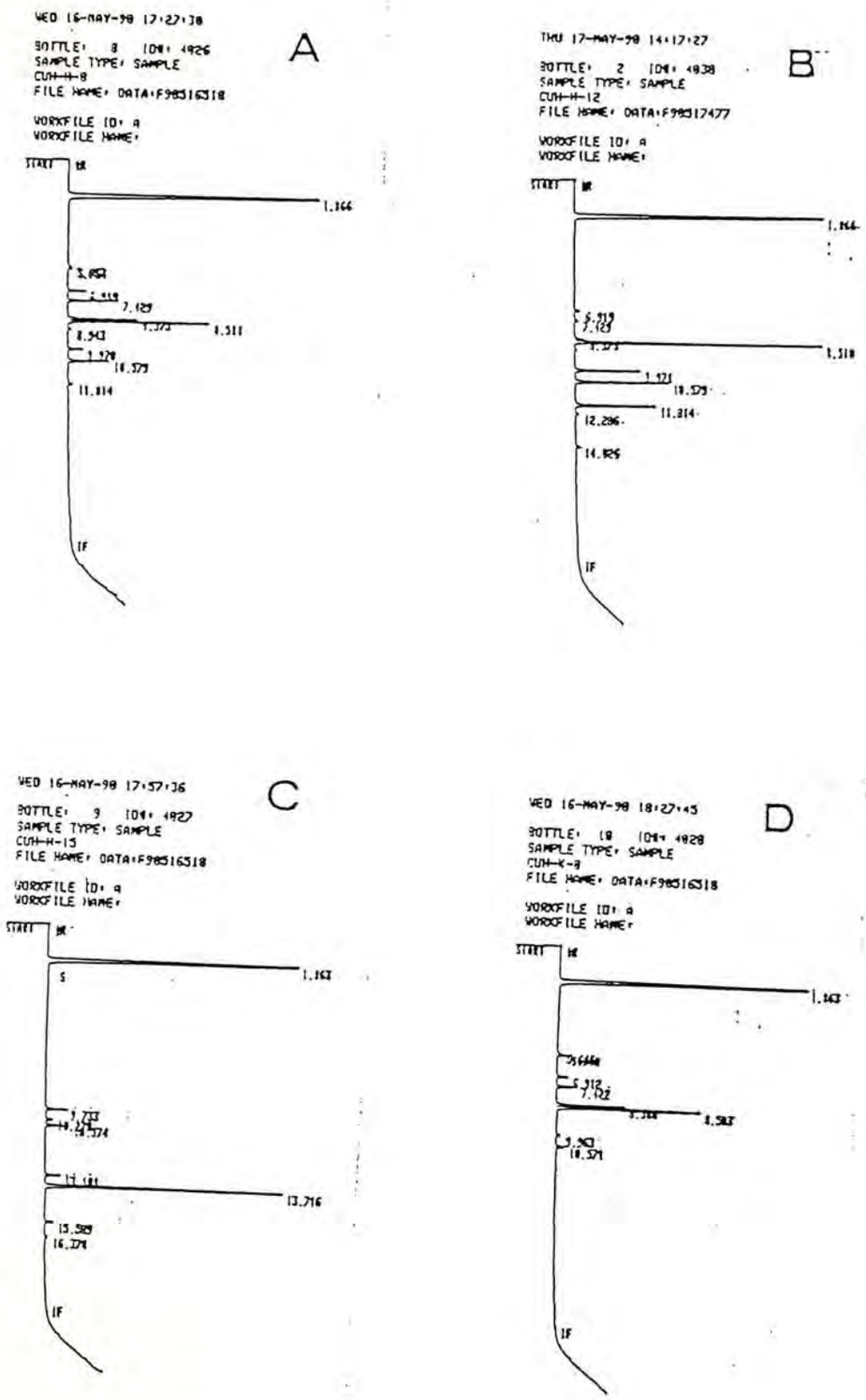


Figure 12: Fatty acids profiles of the identified strains. (A) H-8, (B) H-12, (C), H-15, (D) K-8.

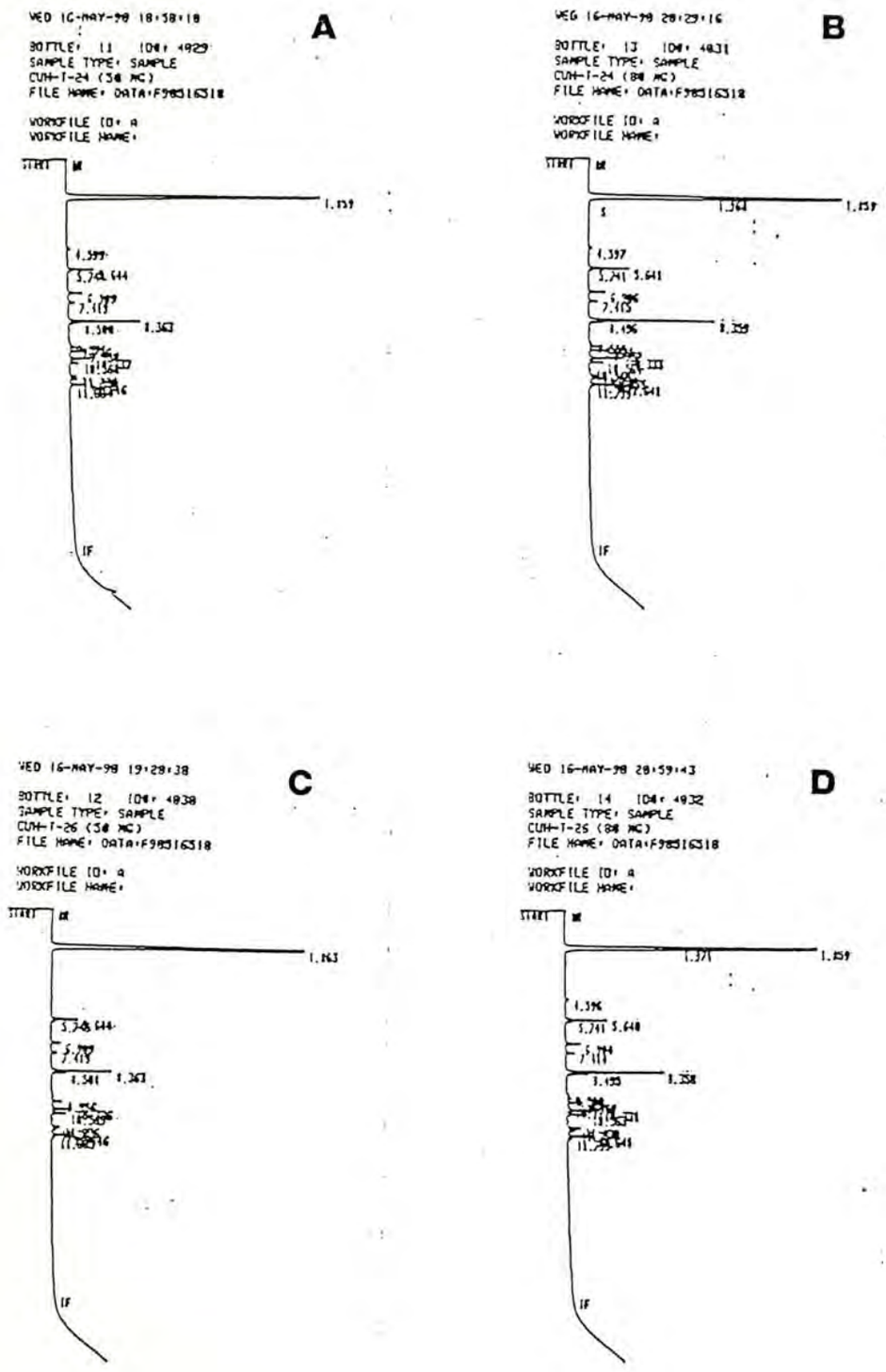
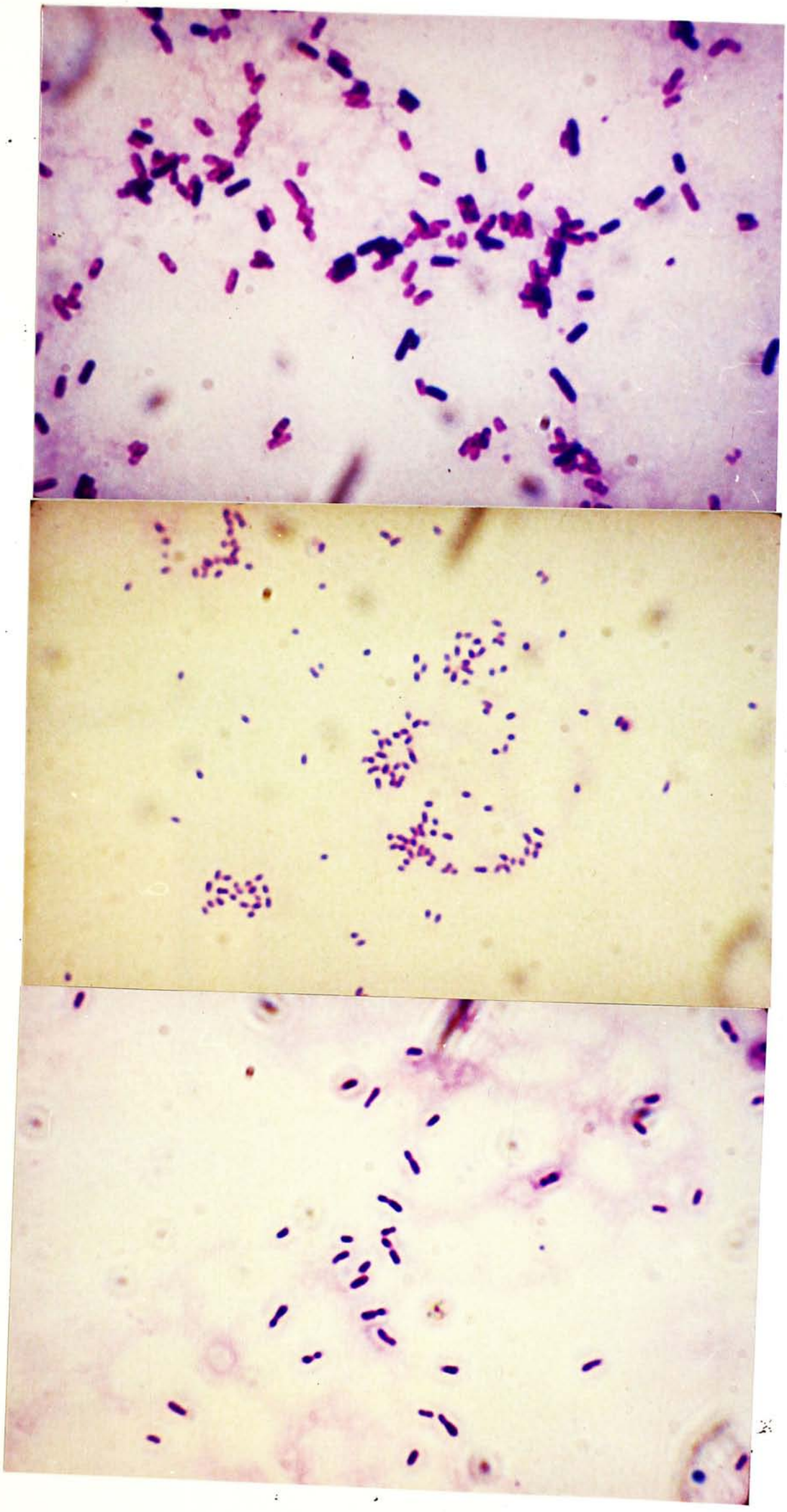


Figure 13: Fatty acids profiles of the identified strains. (A)T-24 50mg, (B) T-24 80mg, (C) T-26 50mg, (D) T-26 80mg.



A

B

C

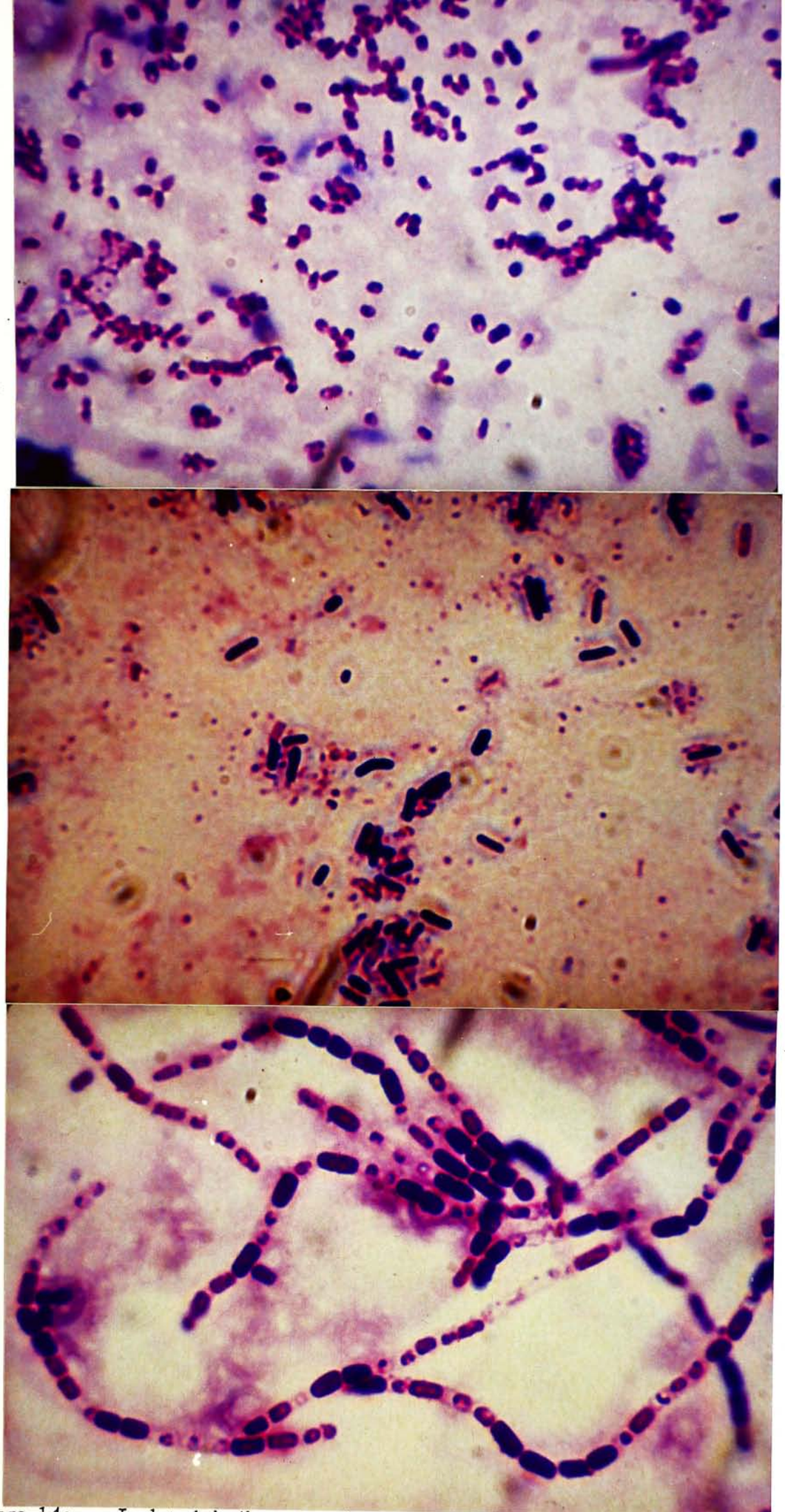


Figure 14: Isolated indigo carmine degrading strains under microscope (magnification = 1,000X), stained by crystal violet. (A) H-8, (B) H-12, (C) H-15, (D) K-8, (E) T-24, (F) T-26.

(Figure 12&13). Isolate K-8 and H-12 were identified as *Micrococcus* species. Isolate T-24 and T-26 were *Bacillus* species. In order to identify the *Bacillus* species, it was necessary to run two cell mass. The other two strains, H-15 and H-8, were *Agrobacterium* and *Bacillus* respectively.

IV. Characterization of the batch culture of *Micrococcus* sp. H-12

A. Inducibility of decolorization ability

When the culture was supplied with indigo carmine during incubation, the adapted cells could decolorize 25.3% of dye in the assay (Table 2). However, if the culture was free of dye, the bacterial population would be non-adapted to the dye and the cells could only decolorize 6.3% of indigo carmine consequently. There was about four fold difference in the decolorization ability between the adapted and non-adapted resting cells.

B. Effect of temperature

When comparing the growth curves of strain H-12 in 30°C and 37°C, it was found that strain H-12 had a slightly higher specific growth rate in the log phase at 37°C than 30°C (Figure 15). Thus, it built up its population more quickly and reached the stationary phase earlier. It took about 15 hours in 37°C and 25 hours in 30°C respectively. However, the final bacterial population at the stationary phase did not have a significant difference. They differed only in the rate of establishing the population. With respect to the decolorization rate, culture in 37°C was found to be

Table 2 : Comparison of the decolorization ability of the resting cells of strain H-12 in culture of with (+) and without (-) indigo carmine.

INDIGO CARMINE IN CULTURE	PERCENTAGE OF DECOLORIZATION (%)
+	25.3
-	6.3

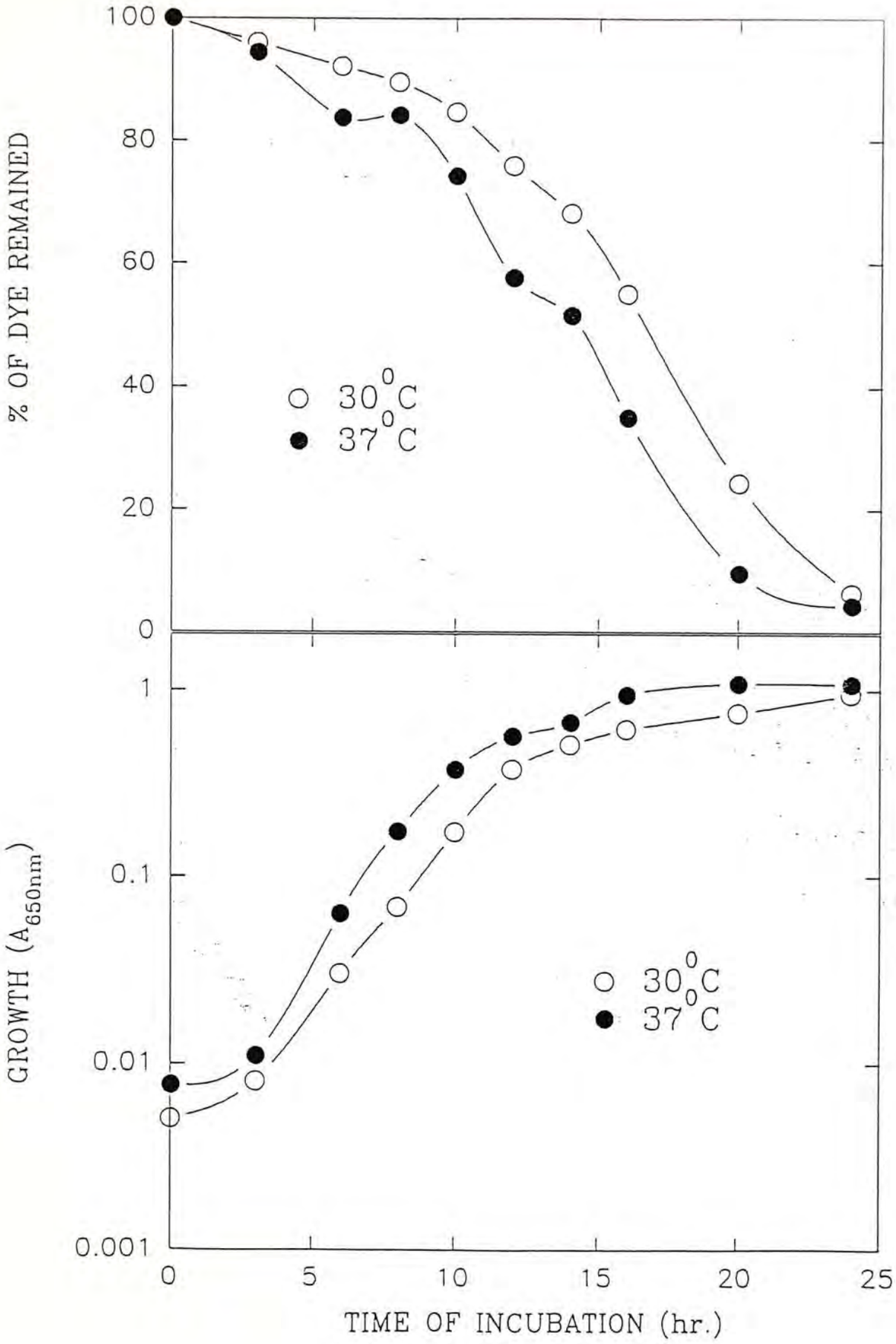


Figure 15: Effect of temperature on the growth and decolorization of indigo carmine by strain H-12.

more efficient. The time for 50% decolorization at 37°C was 14 hours while 16 hours at 30°C. Nevertheless, the decolorization could proceed to complete elimination of the dye at the end of the experiment.

On the other hand, strain H-12 had no observable growth on the I.M. plate at 55°C. It was unlikely to be thermophilic.

C. Effect of pH

In the range of pH 6.0-8.0, pH 6.5 was the optimum for the bacterial growth (Figure 16, Table 3). In this pH, the bacterial population obtained the highest specific growth rate (0.3822 hr^{-1}), the greatest final cell density (absorbance of 1.2437 at 650 nm) and shorter time to decolorized the dye (time for 50% decolorization = 9 hrs.). Strain H-12 grew more poorly at more alkaline pH and was the worst in pH 8.0. There was two fold difference between pH 6.5 and 8.0 in the specific growth rate and final cell density. The time for decolorization was greatly hindered at pH 8.0 (time for 50% decolorization=16 hrs.).

D. Effect of aeration

Growth of strain H-12 was much more extensive in shaking culture than static one (Figure 17). The final cell density in static culture was about one-fifth of the shaking one. It was 0.2223 in absorbance at 650 nm in the static culture compared with 1.0781 in shaking culture. However, the decolorization ability of the static culture was superior to the shaking one. The maximum specific activities of the static

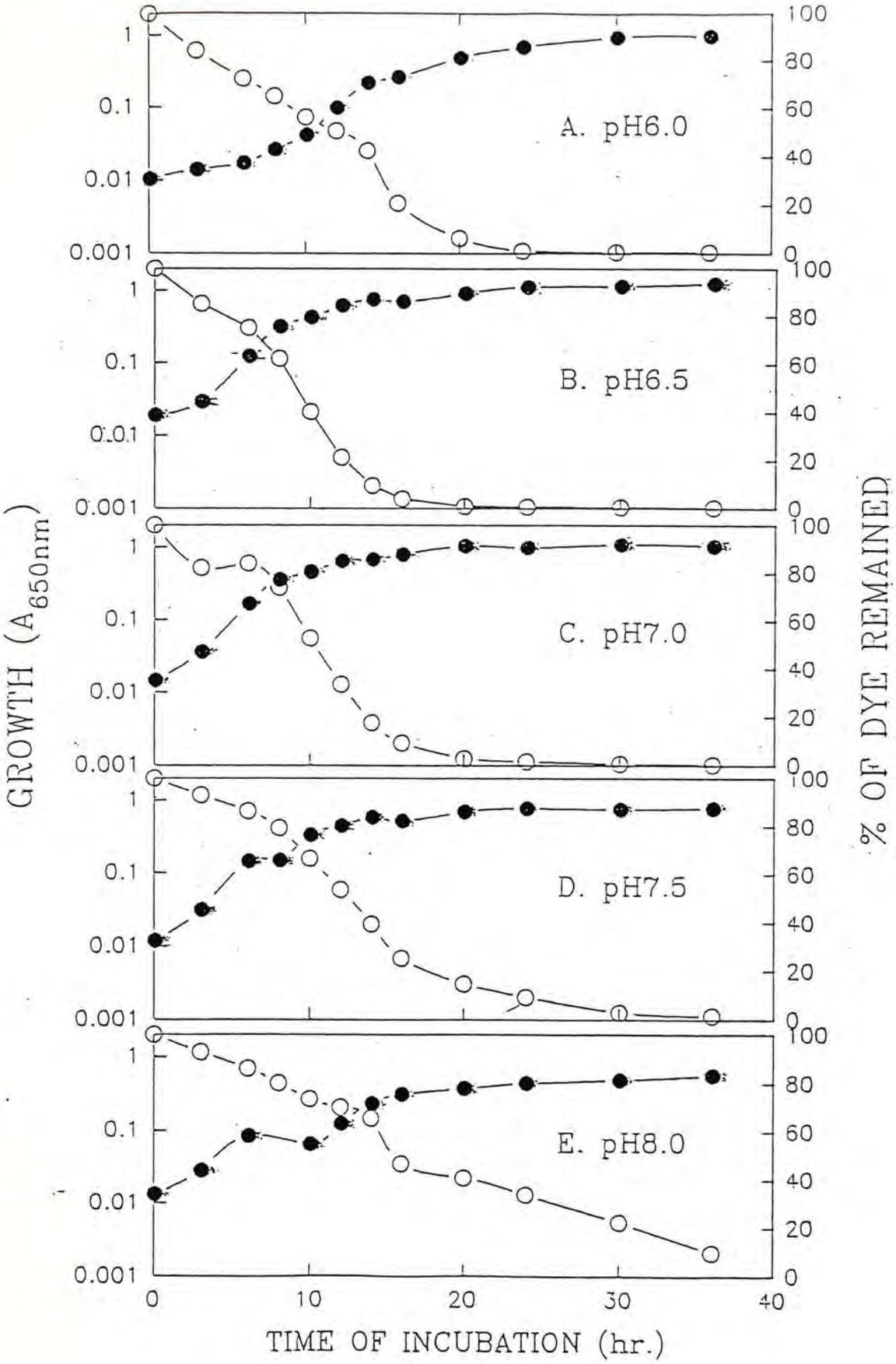


Figure 16: Effect of pH on the growth and decolorization of indigo carmine by strain H-12.

Table 3 : Effect of pH of cultural medium on the growth and decolorization of indigo carmine by strain H-12.

pH	μ (hr^{-1})	t_a (hr.)	D (A_{650nm})	% left (%)	Decol. t_b (hr.)
6.0	0.3164	20.5	0.9800	0	11
6.5	0.3822	12.0	1.2437	0	9
7.0	0.3630	10.5	1.0327	0	10
7.5	0.3380	10.5	0.7796	0	13
8.0	0.1909	15.5	0.5657	9.7	16

μ = specific growth rate
t_a = time for 50% growth of the bacterial population
D = cell density at stationary phase
% left = percentage of dye remained at the end of the experiment (36 hrs.)
Decol. t_b = time for 50% decolorization of indigo carmine

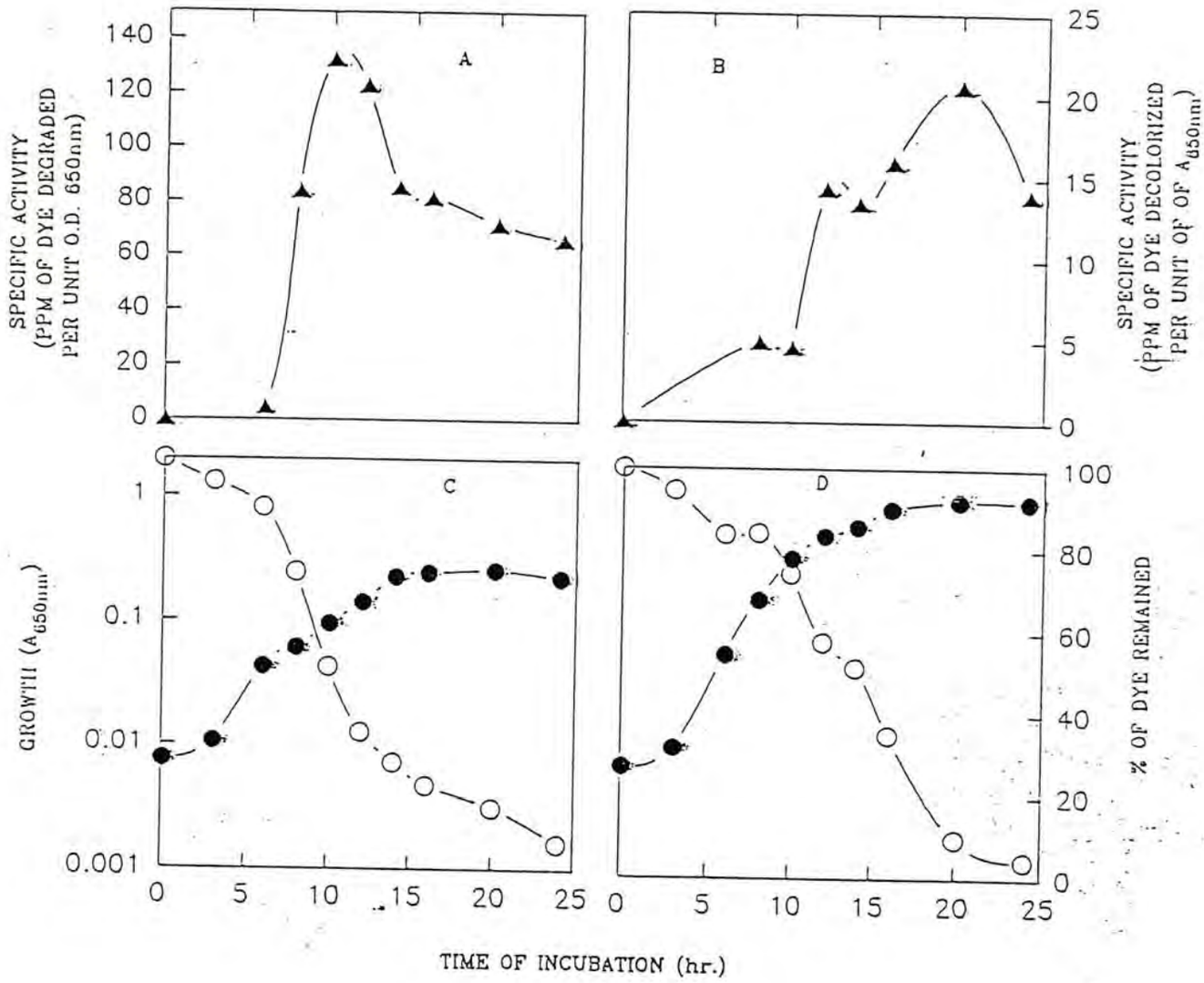


Figure 17: Growth and decolorization of indigo carmine by strain H-12 in static culture (A,C) and shaking culture (B,D).
(● = growth, ○ = decolorization, ▲ = specific activity)

culture was six times that of the shaking one. In the static culture, the maximum value was about 130. Furthermore, the peak of maximum activity appeared at different times under these two conditions. It appeared at the mid-log phase of the static culture but at early stationary phase of the shaking culture. Nevertheless, both cultures could decolorize the dye completely at the end of the incubation (24 hrs.).

E. Growth and decolorization under anaerobic condition

Strain H-12 was able to grow under anaerobic condition. After 48 hours incubation, colony was formed on the I.M. plate. Halo was observed around the colony. However, upon standing in the air, the halo gradually disappeared and returned to the original blue colour of indigo carmine.

F. Effect of carbon sources

Among the five carbon sources being studied, tryptone was the best carbon source for use by strain H-12 in terms of final cell density (Figure 18, Table 4). Glucose gave rise to the greatest growth specific growth rate (0.3748 hr^{-1}) but the final cell density was about the same as starch. Strain H-12 utilized the organic salt as carbon sources much less efficiently. It could only grow to 0.2443 (absorbance at 650 nm) after 48 hours by utilizing acetate and there was only very limited growth in the case of citrate.

Decolorization of indigo carmine followed the same trend of the growth. Decolorization was the most rapid (time for 50% decolorization=12 hrs.) when

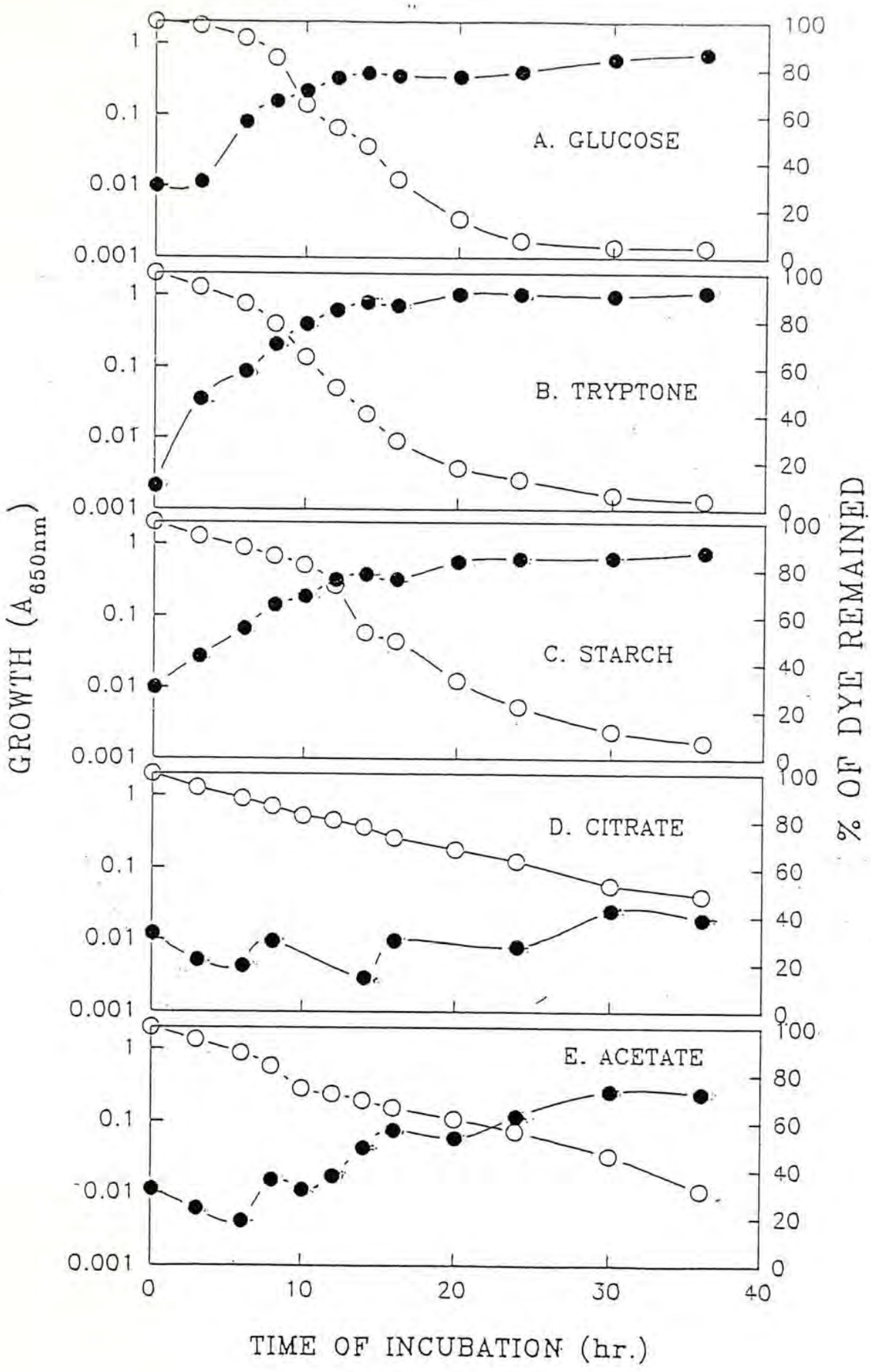


Figure 18: Effect of carbon sources on the growth and decolorization of indigo carmine by strain H-12.
(A) glucose (D) citrate
(B) tryptone (E) acetate
(C) starch
In each case, 1% of the corresponding carbon source was supplied.

Table 4 : Effect of carbon sources on the growth and decolorization of indigo carmine by strain H-12.

Carbon source	μ ($hr.^{-1}$)	t_a (hr.)	D (A_{650nm})	% left (%)	Decol. t_b (hr.)
glucose	0.3748	13.5	0.7274	4.3	14
tryptone	0.3182	11.5	1.1073	3.9	12
starch	0.2742	14.5	0.7713	7.1	16
citrate	N.D.	N.D.	0.0191	48.9	36
acetate	0.1527	25.0	0.2443	31.7	26

μ = specific growth rate
t_a = time for 50% growth of the bacterial population
D = cell density at stationary phase
% left = percentage of dye remained at the end of the experiment (36 hrs.)
Decol. t_b = time for 50% decolorization of indigo carmine
N.D. = not determined

tryptone was supplied as the major carbon source. For citrate, the limited growth caused a delay in the decolorization of the culture. After 36 hours, only 50% of dye was decolorized.

G. Substitution effect of indigo carmine for major carbon source

No major differences with respect to the two growth curves could be observed (Figure 19). Since the yeast extract contained in the cultural medium could serve as a limited carbon source, there was certain growth in the modified I.M. without the dye and tryptone. The addition of indigo carmine caused no obvious difference in the growth. At the end of 36 hours, both cultures reached a cell density of around 0.2 (absorbance at 650 nm).

Decolorization occurred rapidly during the log phase in the bacterial culture but became level off in the stationary phase. At the end of the experiment, 75% of the dye was eliminated by the culture.

H. Substitution effect of indigo carmine for major nitrogen source

Bacterial population of strain H-12 could reach a greater final cell density when indigo carmine was supplied to replace ammonium chloride (Figure 20). When totally 100 ppm of the dye was supplied, the stationary cell density was 0.7341 in absorbance at 650 nm compared with 0.595 in the absence of the dye. There was 18.9% increase in cell density. In the presence of the dye, the bacterial population could achieve a faster growth rate at the log phase and finally lead to a greater

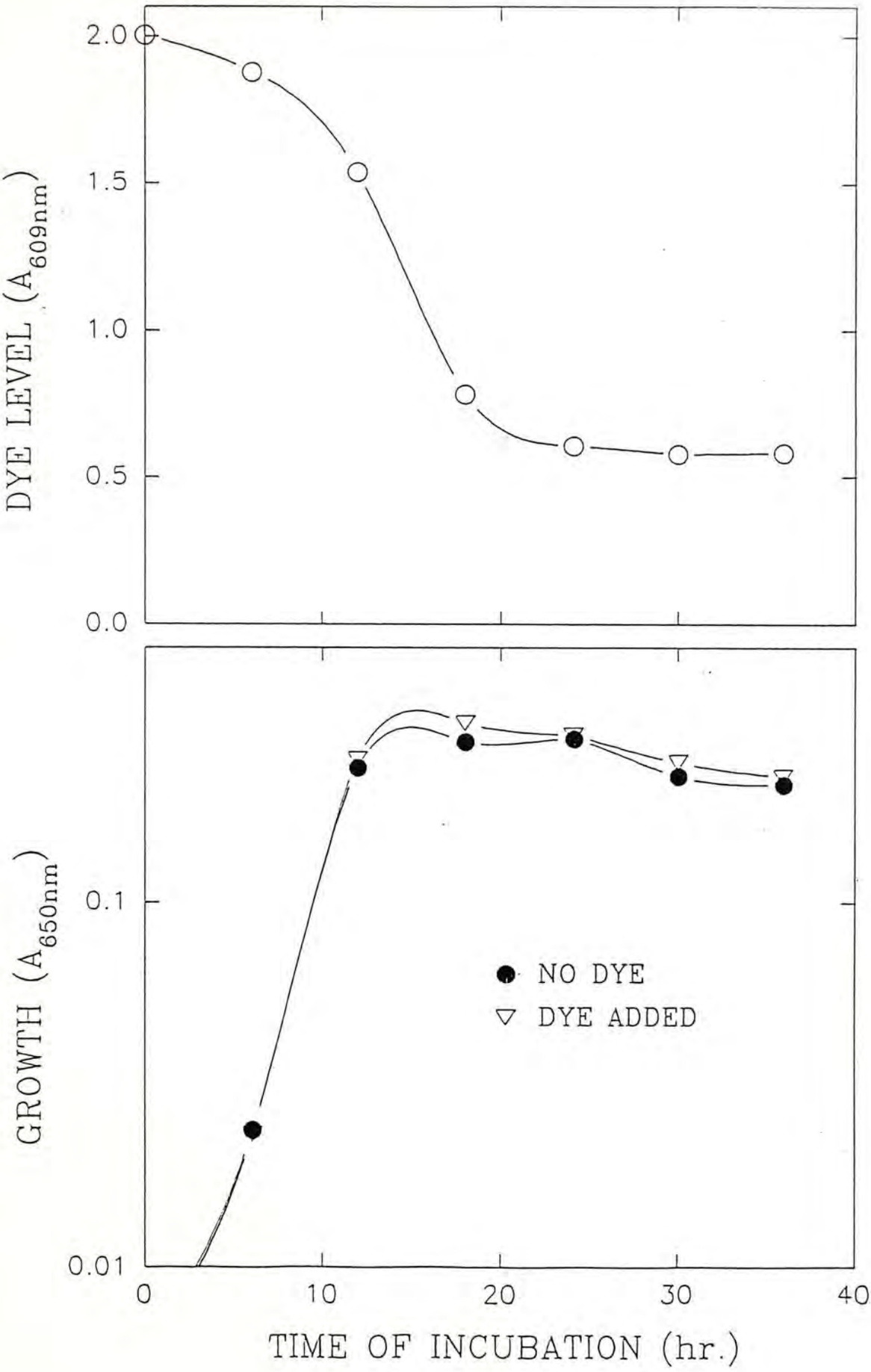


Figure 19: Substitution effect of indigo carmine to replace typtone in I.M..

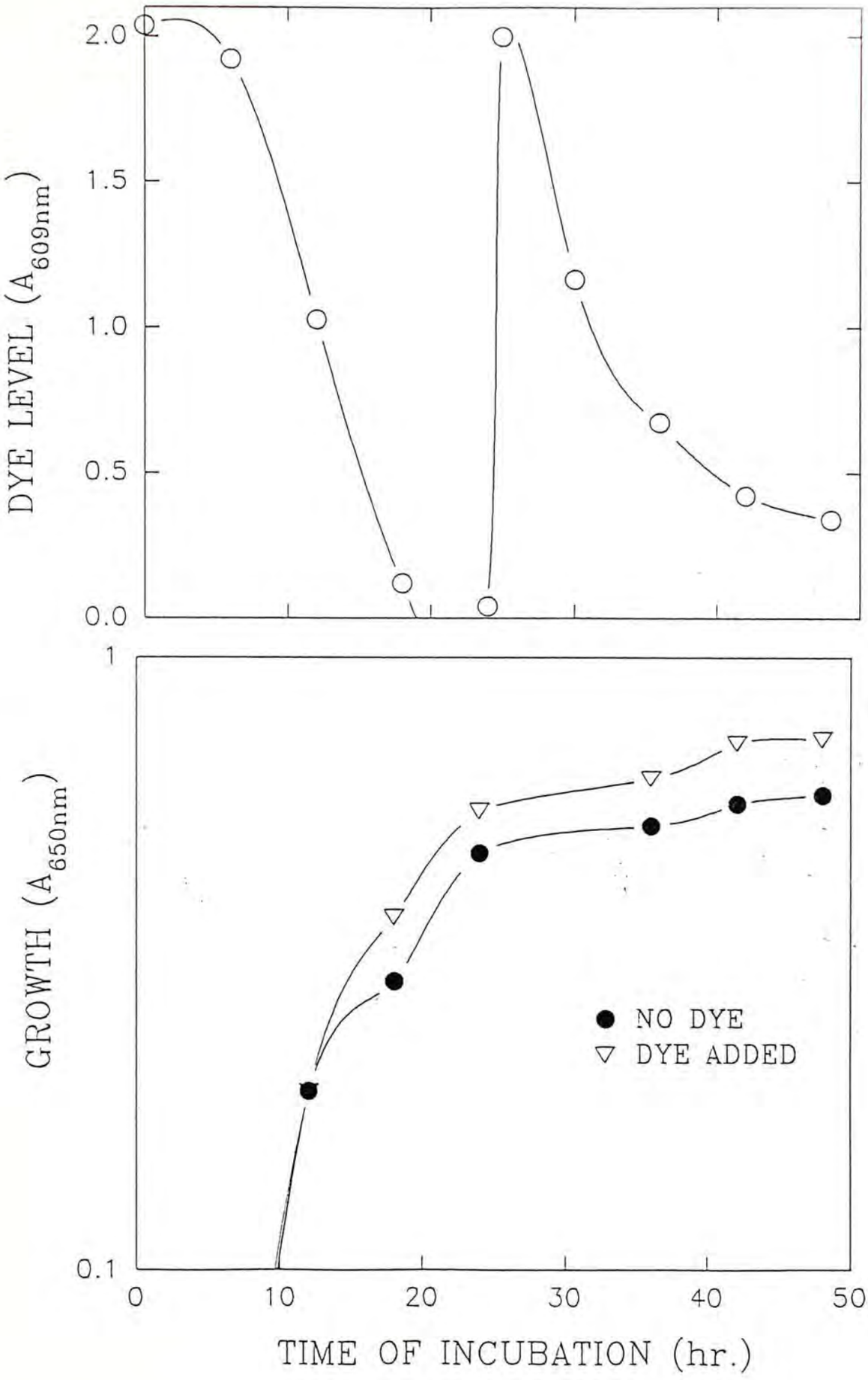


Figure 20: Substitution effect of indigo carmine to replace ammonium chloride in I.M.. In addition, glucose was supplied to replace typtone as carbon source.

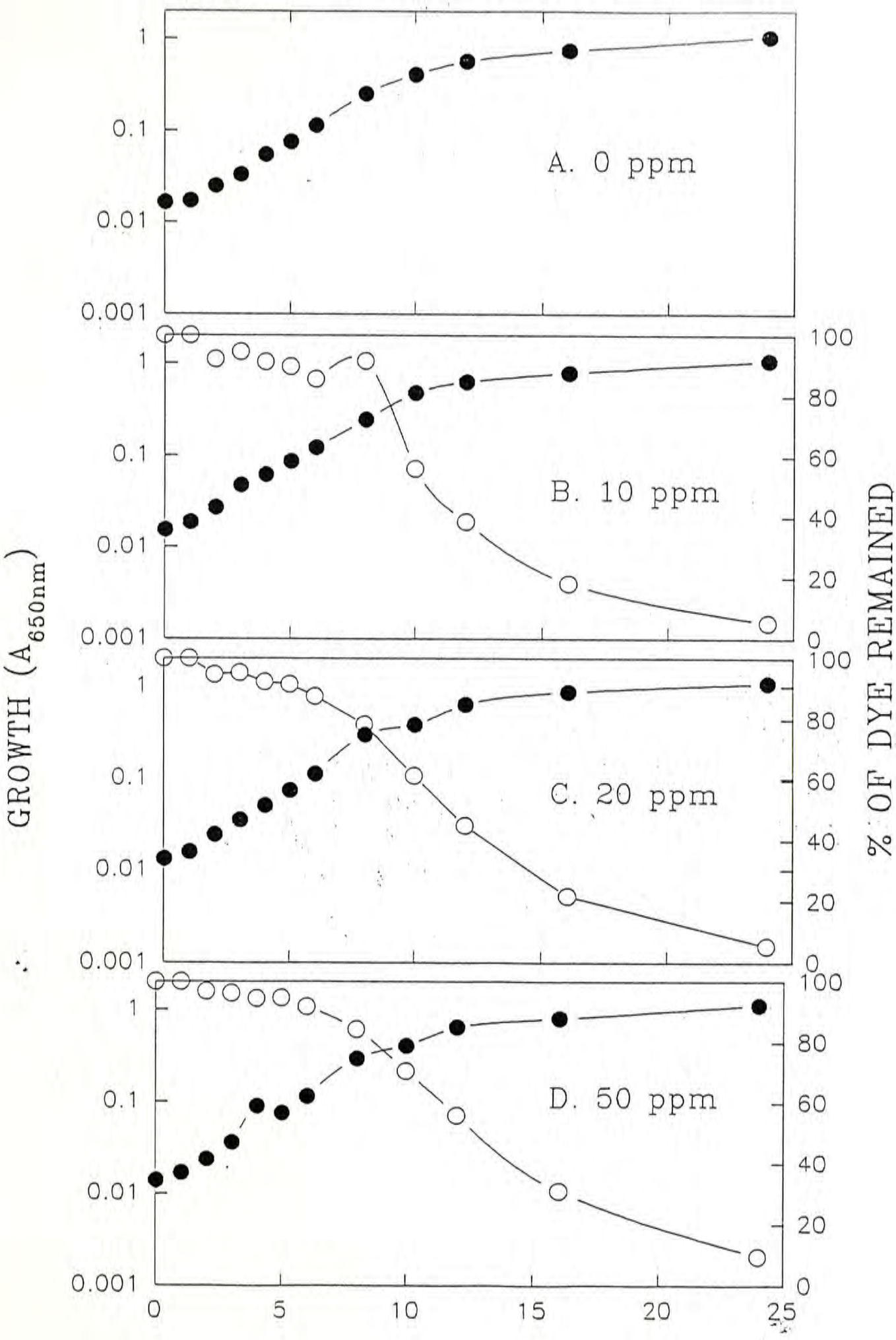
bacterial population at the stationary phase.

At the beginning of the experiment, 50 ppm indigo carmine was supplied to the culture and was decolorized completely at the first 24 hours. It could be deduced from the drop in the absorbance of the supernatant. At the 24th hour, another 50 ppm indigo carmine was added. The dye was decolorized very efficiently by the bacterial population. There were two cycles of decolorization observed in the incubation period.

I. Evaluation of inhibitory effect of indigo carmine

The growth of strain H-12 did not seem to be affected significantly by indigo carmine up to 500 ppm (Figure 21, Table 5). In the absence of the dye, the specific growth rate of strain H-12 was 0.1341 hr^{-1}. The specific growth rate at higher concentrations of the dye did not have any obvious change. According to the growth inhibition calculated, only 4.0% inhibition was observed at 500 ppm. At the same time, the final cell densities in various concentrations of indigo carmine were essentially the same.

Although the growth of the bacterial population was not affected by the high concentrations of the dye, the decolorization curves were different. The time for 50% decolorization increased with the concentration of the dye. At 10 ppm, the time was 10.5 hours, while at 500 ppm, 20.5 hours was needed to decolorize 50% dye. Moreover, the percentage of dye remained in the culture increased at high concentrations of indigo carmine.


A. 0 ppm
B. 10 ppm
C. 20 ppm
D. 50 ppm
GROWTH (A_{650nm})
% OF DYE REMAINED
1
0.1
0.01
0.001
100
80
60
40
20
0
0
5
10
15
20
25

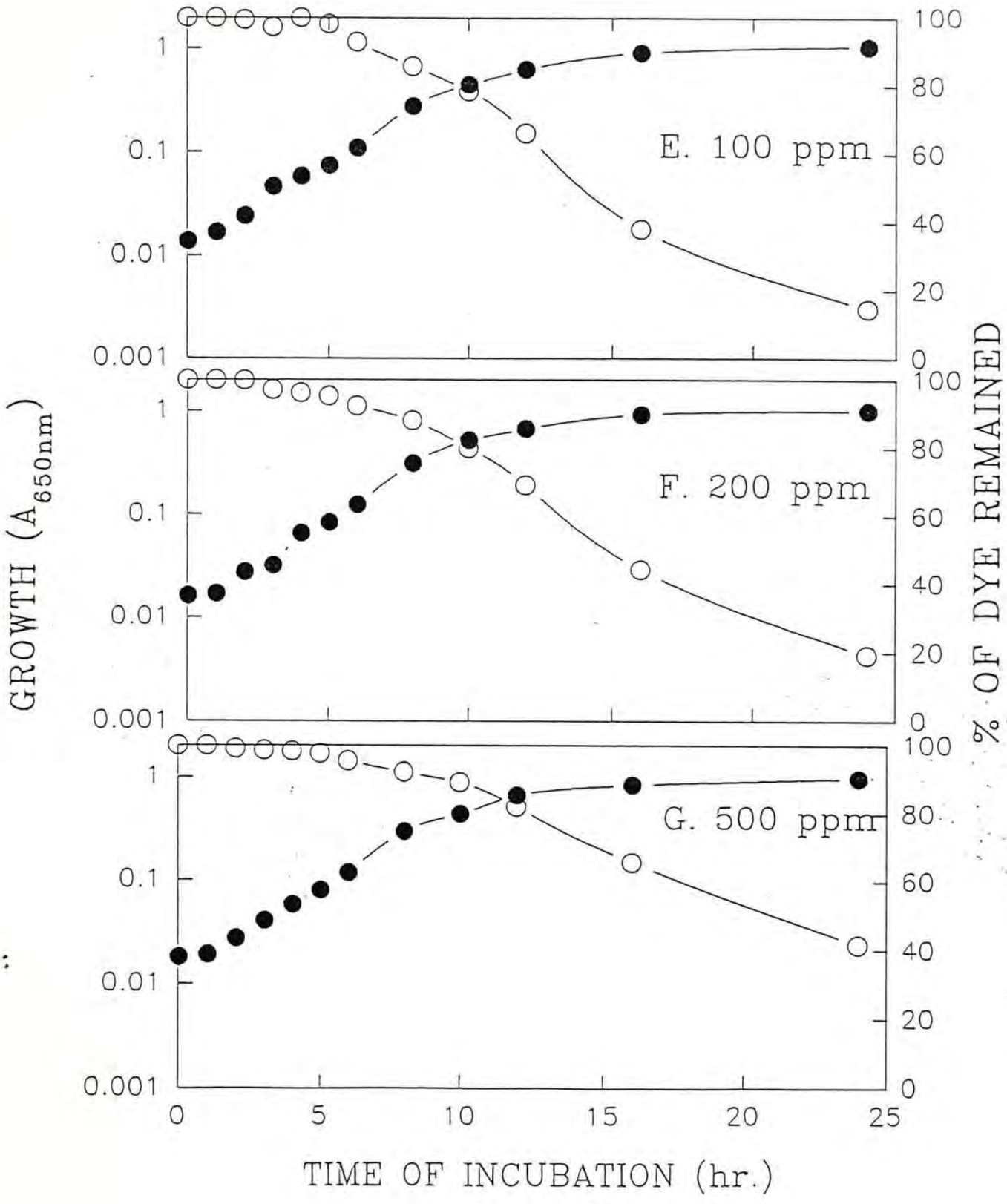


Figure 21: Inhibitory effect of indigo carmine on the growth and decolorization ability of strain H-12.
(A) 0 ppm (E) 100 ppm
(B) 10 ppm (F) 200 ppm
(C) 20 ppm (G) 500 ppm
(D) 50 ppm

Table 5 : Inhibitory effect of different concentrations of indigo carmine on the growth and decolorization ability of strain H-12.

carmine conc. (ppm)	μ ($hr.^{-1}$)	t_a (hr.)	D (A_{650nm})	G.I. (%)	% left (%)	Decol. t_b (hr.)
0	0.1341	11.0	1.0436	-	-	-
10	0.1447	10.5	1.0604	0	5.2	10.5
20	0.1336	11.0	1.0753	0.4	5.4	11.0
50	0.1257	11.0	1.1077	2.4	9.6	12.5
100	0.1437	10.5	1.0526	0	14.6	14.0
200	0.1360	10.0	1.0026	0	19.2	14.5
500	0.1287	10.5	0.9679	4.0	41.6	20.5

μ = specific growth rate
t_a = time for 50% growth of the bacterial population
D = cell density at stationary phase
G.I. = growth inhibition

$$\frac{\mu \text{ (no dye)} - \mu \text{ (dye)}}{\mu \text{ (no dye)}} \times 100\%$$

% left = percentage of dye remained at the end of the experiment (24 hrs.)
Decol. t_b = time for 50% decolorization of indigo carmine

V. Characterization of the resting cells of strain H-12

A. Effect of temperature

In the temperature range of 0-60°C, the resting cells had the highest activity at 37°C (Figure 22). At 37°C, 26.2% of dye was decolorized. The activity dropped readily towards higher or lower temperature. At the highest temperature in this experiment, 60°C, the resting cells still remained the activity to degrade 20% of dye while activity was the lowest at 4°C at which only 11.0% of dye was decolorized.

B. Effect of pH

Study on the effect of pH on the resting cells revealed the optimum pH at 4.0 (Figure 23). The drop in activity at higher or lower pH was drastic. The decolorization activity was low at pH greater than 7.0. At the optimum pH, the resting cells were able to degrade 55% of dye.

C. Effect of aeration

In both conditions, static and shaking, the activity of the cells was the greatest at the first hour (Figure 24). After that, the activity gradually decreased. The static incubation did not differ so much from the shaking one. Although it was slightly higher at all time intervals, the greatest difference did not exceed 2%. After 6 hours incubation, 13.05% of dye was degraded in the static incubation while 11.42% in the case of shaking incubation.

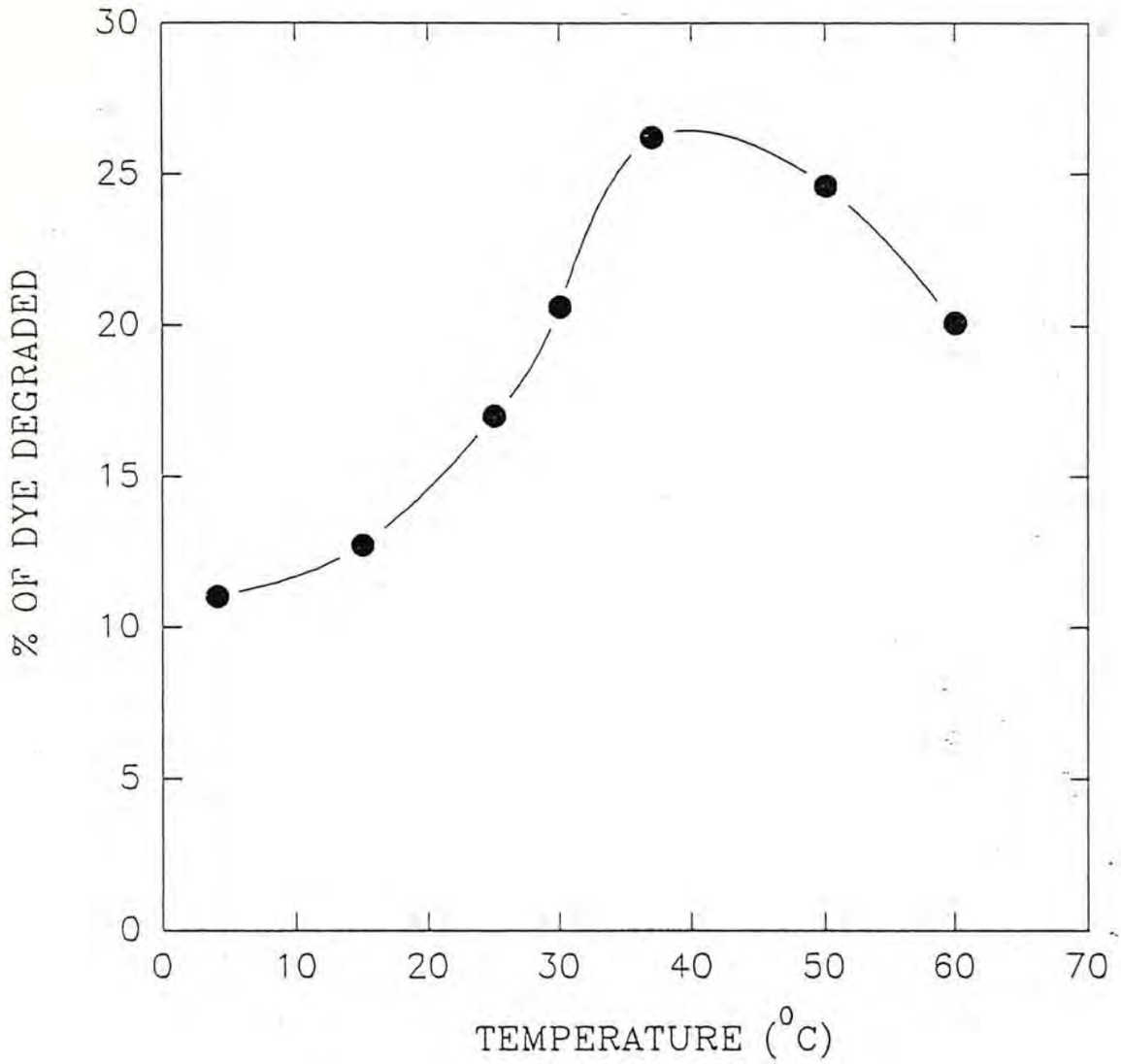


Figure 22: Effect of temperature on the decolorization of indigo carmine by the resting cells of strain H-12.

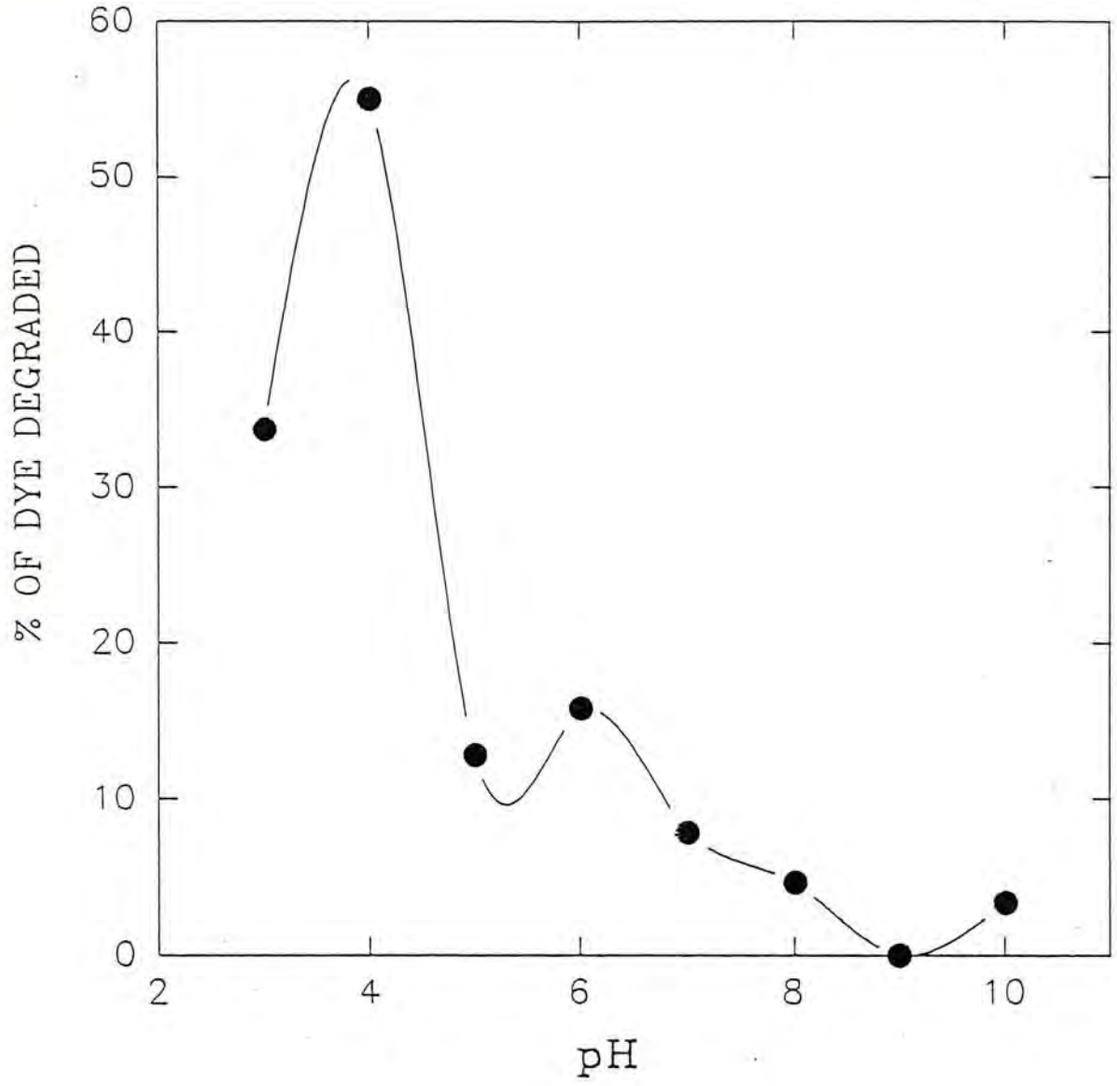


Figure 23: Effect of pH on the decolorization of indigo carmine by the resting cells of strain H-12.

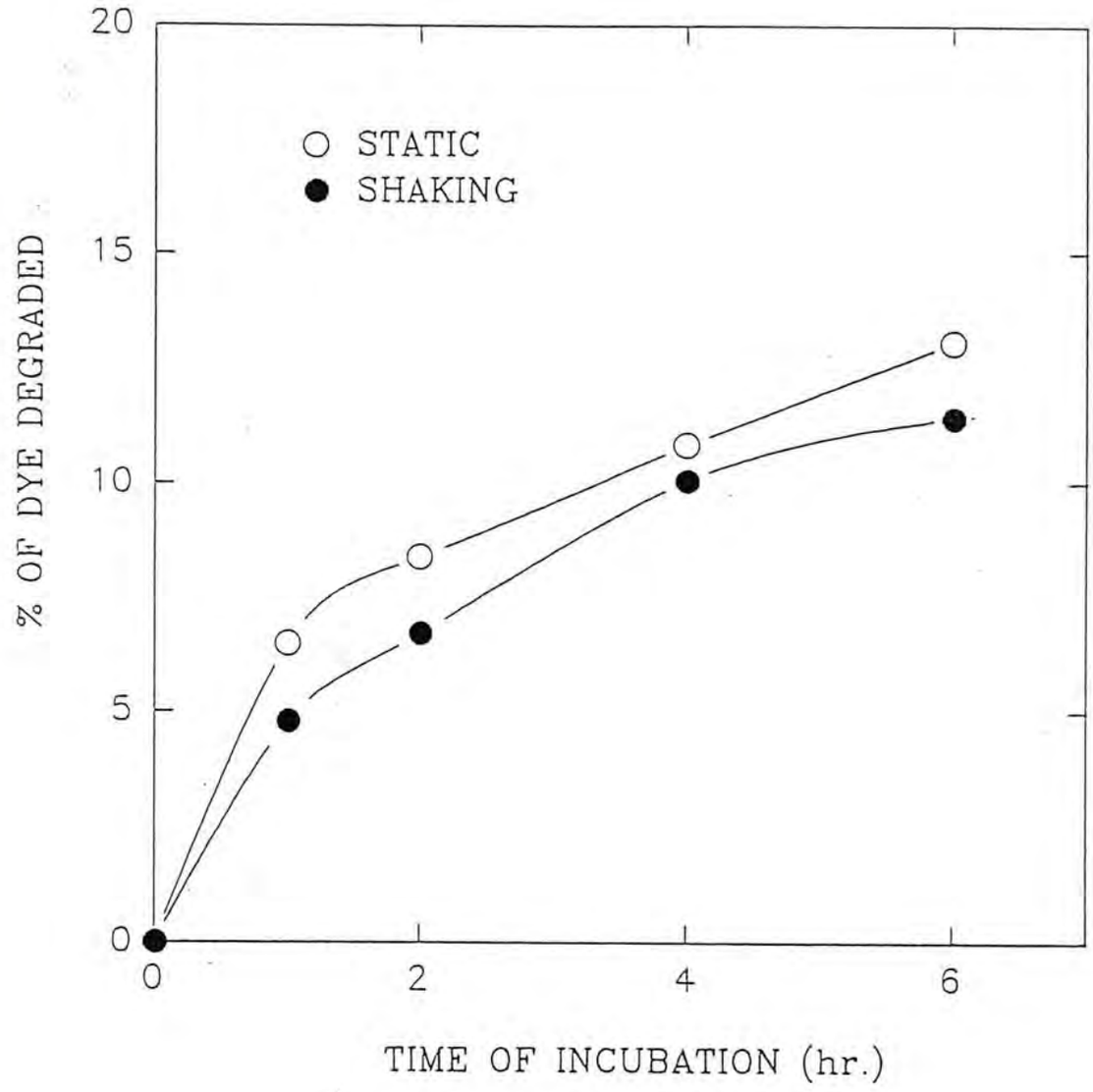


Figure 24: Effect of shaking on the decolorization of indigo carmine by the resting cells of strain H-12.

VI. Decolorization by supernatant and cell free extract

Supernatant of the adapted bacterial culture was found to contain high decolorization abililty towards indigo carmine (Table 6). After one hour incubation, about 60% of dye was decolorized by the supernatant. With respect to the cell free extract prepared by French Press, no significant activity was observed. Only 1.4% of dye was removed by the cell free extract. There was a drastic difference between the decolorization ability of supernatant and that of the cell free extract.

VII. Extraction and identification of the degradation products of indigo carmine

Figure 25 showed the change of scanning profile of 200-700 nm in the cultural medium during 24 hours incubation. Two peaks would be observed in the profile at about 310 and 610 nm respectively. The peak at 610 nm decreased significantly during 24 hours. At the end of the experiment, this peak had disappeared. This corresponded to the complete decolorization of the indigo carmine in the cultural medium. The peak at 310 nm also dropped during the incubation. However, the drop was not so rapidly as that at 610 nm. This absorbance was probably due to the ring compounds in the cultural medium.

When the degradation samples were spotted on the TLC plate using methanol as the mobile phase. Two spots were observed (Figure 26). Their R_f values and appearance under UV were summarised in the Table 7. There was a great difference in their intensities when observed under UV. Spot A was found to be the major spot

Table 6 : Comparison of the decolorization ability of the supernatant and cell free extract from the adapted cells of strain H-12.

	control	exp't	percentage of decolorization (%)
supernatant	0.9499	0.5666	59.6
cell free extract	1.0617	1.0466	1.4

exp't = enzyme preparation added to the reaction mixture
control = distilled water added instead of the enzyme preparation

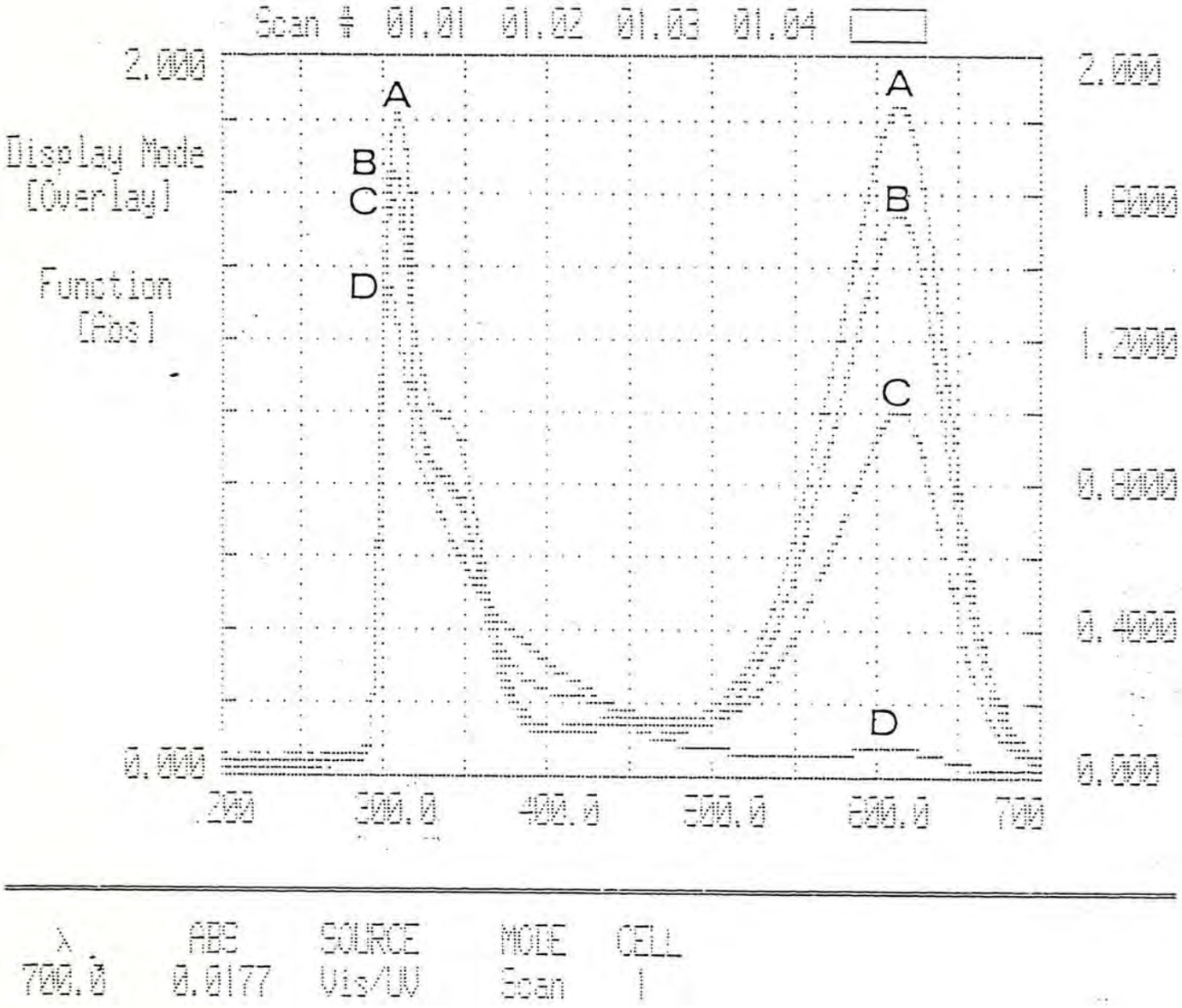


Figure 25: Scanning profile of cultural medium at different time intervals during degradation of indigo carmine by strain H-12. (A) 0 hr.; (B) 6 hrs.; (C) 12 hrs.; (D) 24 hrs.

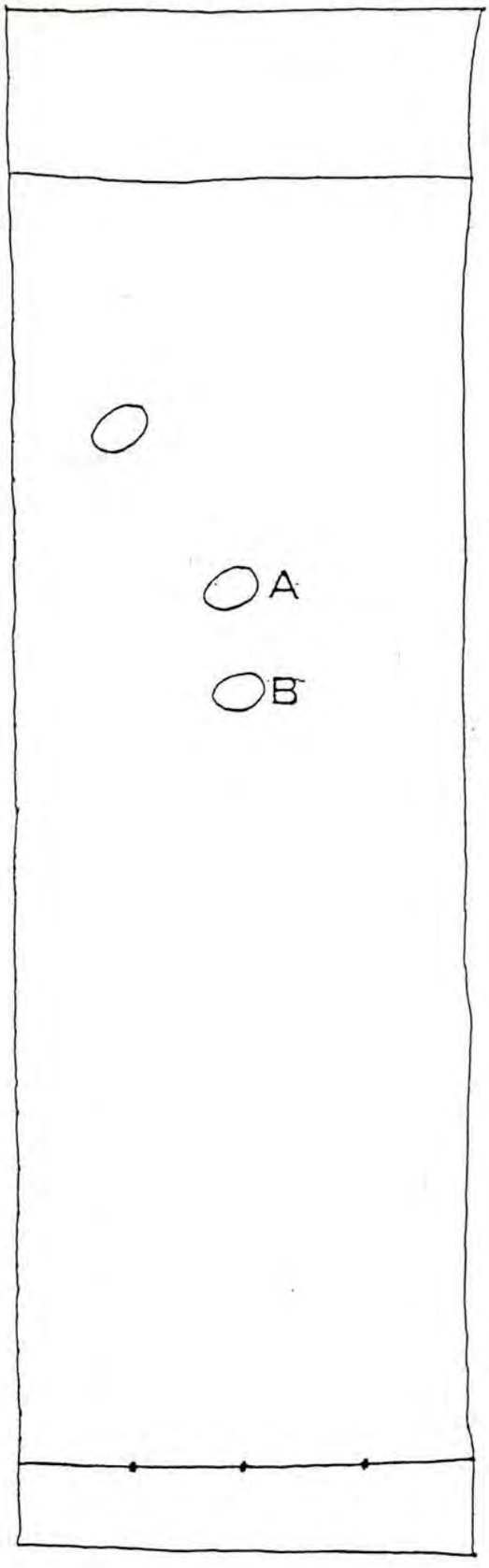


Figure 26: Xerocopy of the result of the separation of degradation products on the TLC plate. Left lane - indigo carmine; central lane - degradation products of indigo carmine by strain H-12 (spots A & B); right lane - extraction of strain H-12 culture without indigo carmine added.

Table 7 : Result of the separation of degradation products of indigo carmine on TLC aluminium sheets silica gel 60 F_{254}.

SPOT	R_f	APPEARANCE UNDER UV	
		SHORT	LONG
A	0.67	Absorption	fluorescent
B	0.59	Absorption	fluorescent

on the plate. These two spots showed absorption at short UV (254 nm) and fluorescence at long UV (366 nm).

The fractions collected after going through the silica gel column according to the method mentioned before were spoted on the TLC plates. Spot A was eluted out of the column from fraction 33 to 43. However, spot B could not be observed clearly on the plate probably due to the low concentration in the preparation.

The purified spot was analyzed by nuclear magnetic resonance (NMR) and mass spectrometry. Figure 27 shows the result of NMR. Peaks A and B were due to the target compound. Other peaks corresponded to the solvents such as water, methanol and DMSO. Peaks A and B had fallen into the range of aromatic compounds and they also represented the presence of two types of proton.

Figure 28 is the result of mass spectrometry. A number of small molecular weight substances were found. These might probably due to the impurities contained in the extraction solvent. In spite of these impurities, a distinct peak was recorded with a corresponding molecular weight of 149 indicating that the major degradative product has a molecular weight of 149 gm/mole.

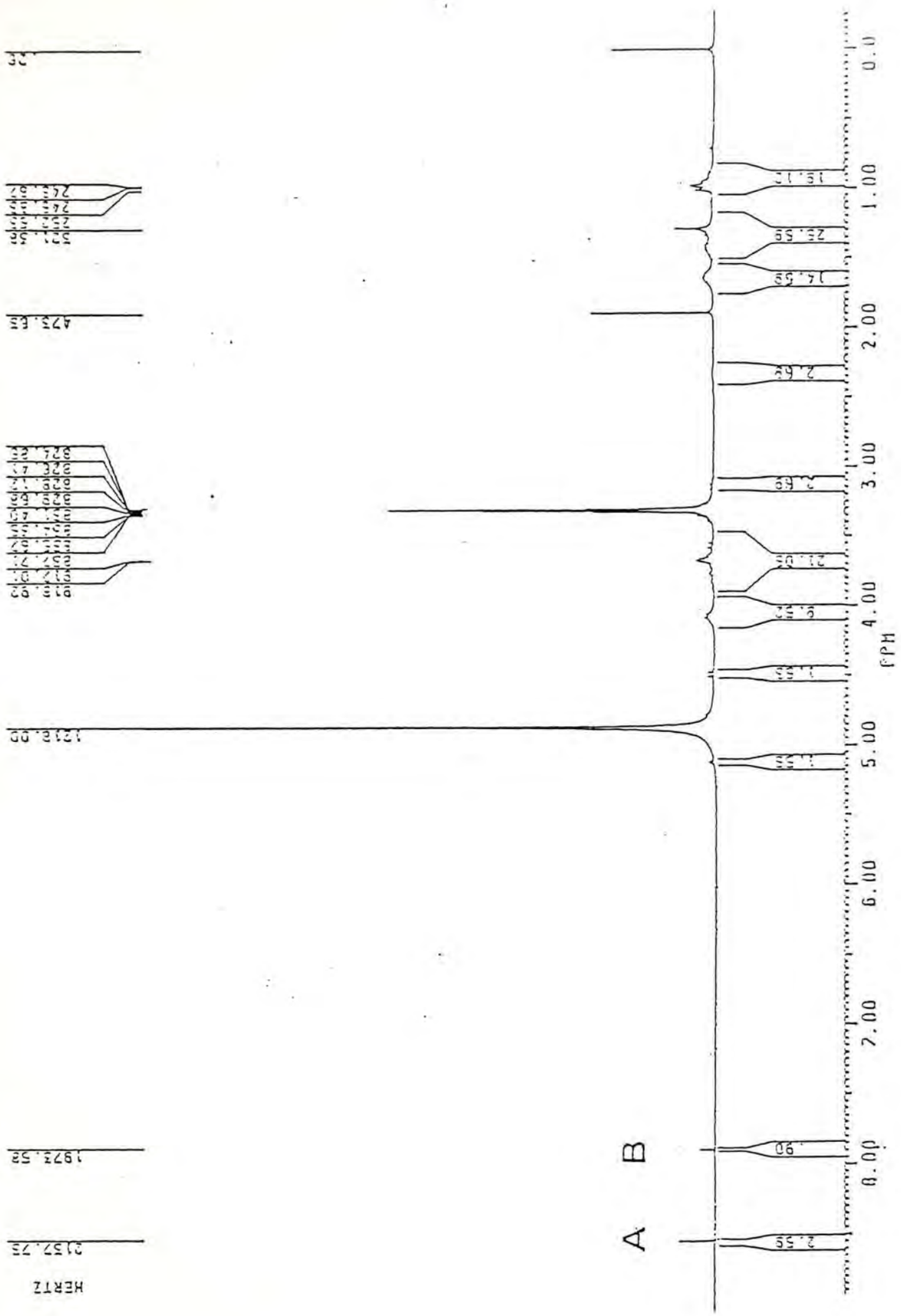


Figure 27: Result of nuclear magnetic resonance (NMR) of degradation product A.

Figure 28: Result of mass spectrometry of degradation product A.

DISCUSSIONS

I. Indigo carmine as the model compound

Among the family of the indigoid compounds, indigo is the most important one according to its share in the market and its extensive use as a dye for denim products in textile industry. However, as it is an insoluble dye, its extremely low solubility in water causes difficulty in studying it at this early stage. Since there is a lack of literature about the biodegradation of indigoid compounds, the difficulty grows even bigger. The reports on the microbial activity on the indigoid compounds are extremely limited. There is not a single reference reporting any micro-organisms able to degrade indigo in the on line computer world-wide literature search for the passed 20 years (1969-1989) and the search for world patent index for the passed 17 years (1963-1980). Besides the biosynthesis of indigo, there were several reports about the reduction of indigo by *Bacillus alkaliphilus*. This strain was isolated by Takahara and Tanabe (1960) and was tried to reduce indigo in the industry fermentation during the dyeing process (Takahara *et al.*, 1961; Takahara *et al.*, 1962; Takahara and Tanabe, 1962). However, it was not a degrading strain.

As there is largely unknown about the biodegradation of indigoid compounds, it is necessary to choose an indigoid compound that can facilitate the study at this very preliminary stage. Among the numerous dyestuff compounds in the indigoid family, indigo carmine is an ideal choice.

Indigo carmine is a derivative of indigo, two sulfonic groups on the parent

molecule of indigo at the 5 and 5' positions. However, this minor derivation had already rendered it to have a much higher solubility in water (1 gm per 100 ml water). But the most important, the substitute groups do not affect the major properties of indigo. For example, indigo carmine remains to appear dark-blue powder with copper luster as indigo. When dissolved in water, indigo carmine solution is blue in colour comparable to the indigo solution when dissolved in organic solvent. For many chemical reactions about the indigoid compounds, the chromophore is usually involved. Grosjean *et al.* (1988) had studied the ozone fading of some indigoid compounds including indigo, dibromoindigo, thioindigo and tetrachlorothioindigo. Results showed that the mechanisms were just the same regardless of the derivatives on the parent molecules since the reaction was determined by the chromophore. As the derivatives on indigo carmine do not affect the chemical structure of the chromophore, chemical reactions of indigo carmine should resemble those of other compounds in this dye class.

In addition to a model compound, indigo carmine has its own significance for studies. Indigo carmine (FD&C Blue No.2) was permitted for food use from 1907 with a safe level for man of 181 mg/day). Past reports of the food dyes claimed that indigo carmine had no mutagenic effect on *Salmonella typhimurium* (Auletta *et al.*, 1977; Brown *et al.*, 1978) and *Saccharomyces cerevisiae* (Sankaranarayanan and Murthy, 1979). But, more recent studies discover that indigo carmine is mutagenic to mammalian and plant system. Roychoudhury and Giri (1989) had showed the mitotic aberrations of *Allium cepa* caused by indigo carmine while sister chromatid exchange in bone marrow cells of mice was demonstrated by Giri and Mukherjee (1990). Table 1 has summarised the results of the mutagenic, carcinogenic and

clastogenic effects of indigo carmine in different test systems.

In Japan, researches were done on the treatment of indigo carmine wastes by activated carbon (Tamura *et al.*, 1987a; Tamura *et al.*, 1987b; Tamura *et al.*, 1988). Now, we are trying a different way to solve the same problem --- biodegradation.

II. Isolation and identification of the degrading strains

The isolation of degrading strains from the polluted environment has become a common practice for environmental microbiologists to sort out desirable strains to degrade particular compound (Idaka *et al.*, 1978; Ogawa *et al.*, 1988; So, 1989). The polluted environment has provided a selective pressure for the desirable degrading strains. Therefore, it is a simple method for isolation by direct screening from the contaminated soil or water samples. In this study, the same rationale and methodology has been employed.

On the other hand, the use of continuous culture is another useful tool to develop novel degrading strains. By varying the substrate concentration in the continuous culture and allowing sufficient time for the genetic exchange between the individuals in a mixed culture, we can develop strains with different substrate specificity. Kulla (1981) had successful developed stains which were able to degrade carboxylated orange I and carboxylated orange II respectively in the continuous culture. The original strain was able to degrade 4,4'-dicarboxyazobenzene only.

For the isolated strains in the study, most of them are showed to be Gram-

positive. Among the six strains that have the greatest degrading activity, all of them are Gram-positive except H-15. There are three *Bacillus* and two *Micrococcus* strains. The indigo reducing strain isolated by Takahara and Tanabe (1960) was also a *Bacillus* species. We do not know whether there is a correlation between Gram-positive strains and the microbial activity on the indigoid compounds. It is too early to draw a conclusion now since there are still too few stains reported.

III. Characterization of the batch culture

The decolorization ability of strain H-12 was inducible. This can be shown from the great difference between the decolorization ability between adapted and non-adapted bacterial population. The inducibility may imply an inducible enzyme system which is expressed when the substrate is present. However, it is not known whether this is co-metabolism or a specific enzyme system really exists for the degradation of indigoid compounds in strain H-12. The dye, indigo carmine may share some structural similarity with the normal substrate to cause the co-metabolism since it does not contain artificially constructed bonding. On the other hand, as this strain was isolated from the environment highly polluted with indigo, a specific system may have evolved to degrade such compounds.

Strain H-12 has better growth at 37°C than 30°C but no growth at 55°C. It is not surprising to know that it is not thermophilic since the environment for the isolation is not at a temperature high enough to favour the thermophilic strains. However, many strains inhabit in the natural environment usually have their optimum temperature for growth at 30°C but strain H-12 achieves better growth and

decolorization ability at 37°C.

With respect to pH, the growth of strain H-12 is the best at pH6.5. The effect of pH can cause a significant influence in the bacterial population. Strain H-12 is more susceptible to alkaline pH than acidic pH. Significant decrease in specific growth rate and final cell density are observed when the pH of the cultural medium shifts up to pH8.0. The decrease in both cases is two fold when compared with those at optimum pH6.5 for growth.

The effect of aeration is quite different for different strains on the degradation of dyestuffs. For some strains, e.g. *Aeromonas hydrophila* var 24B had a rapid elimination of azo compounds in shaking culture than static one but the extent of elimination is small (Idaka *et al.*, 1978). There was also considerable growth in the shaking culture. In this study, strain H-12 was able to achieve a greater rate of decolorization in the static culture as well as obtained the same extent of decolorization as the shaking one although its cell density was much lower in the static condition. Strain H-12 developed a much higher specific activity when in static condition. This phenomenon is especially preferable in the sewage treatment process. Energy can be saved because no aeration is required and the small cell mass generated during the process has simplified the waste disposal of sludge.

Strain H-12 can decolorize indigo carmine under both aerobic and anaerobic conditions. When it is incubated under anaerobic condition, halo is formed but return to blue colour shortly after being exposed to air. This is not the case under aerobic condition. Halo appears under aerobic condition is stable and would not revert. This

may imply that the decolorization of indigo carmine under anaerobic condition is carried out in another different pathway. Mostly probably, indigo carmine is reduced into leuco compound under anaerobic condition. Reduction would occur when indigo carmine serves as an electron acceptor. The leuco compound is trapped and accumulate. As in the case of azo dyes, anaerobic pathways frequently come to a metabolic standstill (Meyer 1981). The products are accumulated in the system. In aerobic condition, the molecule of indigo carmine is split down and hence, the reversion is impossible.

Strain H-12 has an absolute requirement for yeast extract for growth. Attempt had been made to grow strain H-12 in normal minimal salt medium but failed. Therefore, there is difficulty to test whether indigo carmine can serve as carbon or nitrogen source for growth. Because of this, we bypass the difficulty by modifying the I.M. and look at the effect of indigo carmine on growth of strain H-12 when the major carbon or nitrogen source is removed but substituted by the dye.

In the test of removing carbon source, tryptone was replaced by indigo carmine. We cannot see any difference in the growth no matter the dye is added or not. It can be deduced that indigo carmine cannot promote the bacterial growth by serving as extra carbon source to replace tryptone which is a rich carbon source.

According to the chemical structure of indigo carmine, it contains two nitrogen atoms for each molecule. Thus, it is tested for the effect on growth of strain H-12 when the major nitrogen source, ammonium chloride, is absent from the I.M. . The result showed that indigo carmine can promote bacterial growth if ammonium chloride

is not supplied in the culture. The effect on growth is quite evident since 100 ppm of indigo carmine can increases the bacterial growth by 18.9%. Probably, this is due to the metabolism of the dye into usable nitrogen source for the utilization of the population.

In this study , we can only compare the relative growth. To further confirm the above conclusion, we must find out the growth factor needed by this strain and derive a minimal salt medium so that the yeast extract can be completely removed from the medium. Yeast extract has caused interference to the experiment since it alone can serve as carbon and nitrogen sources for limited growth of strain H-12. As a result, we cannot obtain an all-or-none growth pattern to distinguish the effect of the presence of the dye. We have tried to test for the growth factors required but the results are ambiguous. Perhaps, strain H-12 is unable to synthesize more than one growth factors.

Carbon sources are important in affecting the growth and decolorization of indigo carmine by strain H-12. Among the carbon sources being studied, glucose is the most efficient source for strain H-12 that the specific growth rate is the greatest. Glucose has been the central metabolic substrate in the bacterial metabolism. However, the highest bacterial concentration was built up when tryptone was supplied. Strain H-12 has a better metabolic ability on the complex organic carbon source than the organic salt. Strain H-12 can obtain a fairly good growth by using starch. This is an exceptional advantage for further development for use in sewage treatment. As the dyeing waste water usually contains a high level of starch which comes from the textile processes, the ability to use starch can enhance the

maintenance of the bacterial population as well as removing starch in the wastewater at the same time. Strain H-12 cannot utilize citrate as carbon source efficiently. It showed limited growth when citrate was supplied instead of tryptone. The inability of strain H-12 to use citrate may be due to the lack of appropriate transport protein on cell membrane. Although strain H-12 can utilize acetate for growth, the final bacterial population is quite small compared with the complex carbon source.

The preference for strain H-12 to use complex organic carbon sources may be related to the environment from which it is isolated. The original habitat of strain H-12 is filled with complex organic matter in the dyeing wastewater and the organic salts such as citrate and acetate seldom exist. Selection pressure will drive strain H-12 to develop better ability to utilize the complex organic carbon source. This can increase the competitive power of strain H-12 for survival among the mixed population in the environment.

The inhibitory effects of dyestuff on activity and growth of microorganisms usually occur at a higher dye concentration. but some cationic species especially the triphenylmethanes dyes are particularly toxic. Ogawa *et al.* (1988) had studied the growth inhibition of *Bacillus subtilis* (IFO3022) by basic dyes. They found that the inhibition effect of triphenylmethane dyes were evident that less than 10^{-4} mol/l could cause 50% inhibition of growth. It is well known that triphenylmethane has strong sterilizing properties.

Indigoid dyes are not classified as toxic to microorganisms. In a concentration

as high as 500 ppm of indigo carmine, strain H-12 does not show any significant inhibition in the growth. Its high tolerance is definitely an advantage in the sewage treatment process. After incubation for 24 hours, over 90% of dye could be removed in the concentration of 50 ppm. When compared with the activated carbon, the decolorization by strain H-12 is much economic. The activated carbon is a costly material and the recovery after adsorption causes another problem. Decolorization of indigo carmine by strain H-12 can simplify the operation. Under static condition, strain H-12 can maintain a high decolorization ability but a low cell population is built up. This can minimize the need for sludge disposal. Thus, the treatment of indigo carmine waste by biodegradation using strain H-12 is a better method than adsorption by activated carbon.

IV. Characterization of the resting cells

The characterization of the decolorization ability of the resting cells is important since immobilization of resting cells is potential to be developed for applications with higher efficiency. The cells would be much more stable after immobilization and so they can withstand a longer period for use. Moreover, immobilization can increase the effective concentration of the bacterial population to enhance the rate of degradation. The removal of bacterial cells after the treatment also become much easier after immobilization. This can prevent the escape of the bacterial cells into the environment to disturb the ecosystem of the surrounding area.

The studies about the resting cells of strain H-12 showed that they have an optimum temperature of 37°C and an optimum pH of 4.0 for the degradation of the

dye. The optimum temperature of the resting cells is just the same as the growing cells. However, there is a great difference in the optimum pH between the two kinds of cells. The resting cells favour a much more acidic pH than the growing cells. The explanation may be due to the fact that the optimum pH required for cell growth may sometimes deviate from that required for enzymatic activity. During the cell growth, the size of the bacterial population is more important in determining the rate of decolorization.

The resting cells of strain H-12 work equally well in both static and shaking conditions. The rate of decolorization is the greatest at the first hour in both cases. The good performance in the static condition can minimize the cost for aeration and thus more economic in sewage treatment.

For further development, the resting cells can be immobilized into beads which may be further packed into column. This column can be effective in handling dyeing wastewater. The coloured wastewater can be fed into the column. Removal of the dyestuff occurred during the retention within the column. The use of immobilized resting cells is better than growing cells. The conditions applied can be more stringent since no growth is required. This can increase the flexibility of the treatment method.

V. Decolorization by supernatant and cell free extract.

The results have demonstrated that the enzymatic activity of decolorization of indigo carmine locates in the supernatant of the cultural medium rather than within

the cells. The enzymes are thus exoenzymes that secreted out of the cells into the cultural medium. Thus, no enzymatic activity can be recorded in the cell free extract obtained from the disrupted bacterial cells. We have not determined the enzymes are exogenous or endogenous in other five strains being identified yet. Among the five strains, 3 were *Bacillus* species and 2 is *Micrococcus* strain which are all Gram-positive. Gram-positive strains especially *Bacillus* species are known to secrete a variety of exo-enzymes. Therefore, there is a possibility that exo- type enzymes responsible for the decolorization can be found in these strains. We are not sure whether the extracellular nature of the enzymes is correlated with the fact that most of the isolated strains are Gram-positive.

The exo- type nature of the enzymes can facilitate the purification so that the further studies on the enzymology of the decolorization can be much more easier. On the other hand, the purified enzymes can be subjected to immobilization. Enzymes immobilization can prolong the stability of the enzymes than when they exist freely in the cultural medium. Furthermore, the efficiency of the decolorization can be promoted by increasing the effective concentration of the enzymes. In fact, this technology has been tried to be applied in the industrial production of important metabolites.

VI. Extraction and Identification of the degradation products.

As mentioned before, there is no documentation about the biodegradation of any indigoid compounds before. It is a pioneer work on the identification of the degradation products of indigoid compounds.

We have come across with many difficulty in extracting the degradation metabolites. At the beginning, conventional chemical extraction method using chloroform and dichloromethane to extract the I.M. after the growth of strain H-12 was employed. However, due to high content of organic compounds, only the components of the complex medium is extracted.

At the next step, we turned to the use of resting cells. Since there is no known degradation pathway of any indigoid compounds reported. We cannot simply match the degradation products with the authentic compounds by TLC and HPLC. Instead, much greater amount of the degradation products must be gathered for NMR and mass spectrometry analysis. However, the degradation by resting cells is not an efficient way to achieve the above purpose.

Finally, we modified the I.M. by reducing the yeast extract to a minimum and replacing tryptone with glucose. This modification reduced the concentration of the organic compound in the medium and allowed a greater amount of indigo carmine to be added for the growing cells to degrade.

We found that the degradation products cannot be extracted efficiently by non-polar solvent such as chloroform and dichloromethane but only dissolved in methanol. This may probably due to the presence of sulfonic groups to make the molecule much polar.

Two distinctive spots are isolated on the TLC plate. The degradation probably involves the cleavage of the molecule of indigo carmine into two products. As in the

case of azo dyes, the first step in the biodegradation is the cleavage of the azo bonding which is chromophore of the azo dyes. The chromophore is responsible for the different colours appeared in different dye classes. Thus, decolorization of the dyestuffs probably involves the rearrangement of the chromophore. Then, it is expected that the decolorization of indigo carmine requires the breakage of the central carbon-carbon double bond.

According to the intensity of the two spots on TLC, one is the major product. This uneven distribution may imply that one of the two compounds have undergone further metabolism.

The results of NMR and mass spectrometry has been compared with some possible compounds using the chemical synthesis pathway and common chemical reactions of indigo carmine as references. However, none of them can be perfectly matched with the results of NMR and mass spectrometry. Therefore, we cannot postulate a definite chemical structure of the purified degradation product at the present. Nevertheless, the results have shed some light about the degradation mechanism. As the compound appears to be aromatic with molecular weight of 149, it is likely that the degradation of indigo carmine involves the cleavage of the cental carbon-carbon double bond as expected. In order to establish the chemical structure of the degradation product, the analysis by ^{13}C NMR is required to identify the number of carbon atoms present on the molecule.

VII. Prospect

Dyestuffs are classified as recalcitrant compounds that are difficult to be degraded. The Colour Index (Allen *et al.*, 1971) lists about 40,000 commercial colorants involving 7,000-8,000 different chemical structures. Among the vast number of dyestuffs, many of them are synthetic compounds containing novel bonding new to the microorganisms. Moreover, as a dye, it has to be stable enough against various chemical reactions. Therefore, we should expect to know that many of the dyestuffs are difficult to be degraded by microorganisms.

Indigo is a natural dye in which it does not contain artificial bonding. However, its insolubility in water make it difficult for microorganisms to act on and hence make it deposit easily on the water courses and sewage pipeline to cause environmental problem. The treatment of insoluble dye is a tough work, but these dyes are in a large proportion in dyestuff production for various industrial uses. Nevertheless, the majority of studies about the biodegradation of dyestuffs are done on azo dyes. The study on indigoid compound is completely ignored.

In this study, some strains with high degradative ability towards indigo carmine are isolated. Preliminary studies indicated that they are also effective to degrade indigo. Some features of strain H-12 make it advantageous for further studies in the development of new sewage treatment method. The features includes the high decolorization ability towards indigo carmine, the high decolorization ability of static culture, the exo- type nature of the enzymes responsible for the decolorization and the ability to use starch as carbon source.

Coloured effluent from the textile industry and dyestuff factories can be treated

by different methods. Yet, most of the operation do not involve biodegradation. At the present, physico-chemical and chemical processes is the most commonly used method. However, the application of these methods alone cannot achieve satisfactory removal of colour from the dyestuff wastes. With the efforts of environmental microbiologists, the application of microbial strains to degrade such recalcitrant compounds is a plausible way to fulfil the needs.

Figure 29 has summarised the effectiveness of chemical, physical and biological methods in the treatment of different dyestuffs wastes. Biodegradation alone is not effective enough to solve the pollution problem of all the dyestuff waste. The strategy for further development is to establish a concerted model of treatment process combining the advantages of physical, chemical and biological methods.

Nowadays, the biological treatment methods are mainly focused on the use of activated sludge (Idaka *et al.*, 1985; Kanekar and Sarnaik, 1991; Robinson, 1989; Shaul *et al.*, 1991; Urushigawa and Yonezawa, 1977; Yang, 1990). With the accumulating knowledge and microbial strains about the biodegradation of the xenobiotics, the use of specialized strains is a new direction for investigation. This technique has been explored for the treatment of particular type of wastes. This approach allows the optimization of the treatment process in specific conditions of pollution so as to enhance the handling of particular wastes. On the other hand, the specialized culture can be added to the activated sludge to improve its removal capability. It can also be a means to maintain the stability of the sewage treatment system in case there is a great increase in the concentration of particular type of waste in the influent.

Dyestuffs in dyeing wastewaters	Color removal by treatment processes					
	Coagulation alum	Activated carbon	Biological	Combination physico-chemical and biological	Ozone	Sludge adsorptions of dyestuffs
Azoic	O	÷	O	÷	÷	-
Reactive	O	÷	O	÷	÷ (s)	O (÷)
Acid	O	÷	O	÷	÷	O
Basic	O	÷ (s)	÷	÷	÷	÷
Disperse	÷	O	O	÷	O	O
Vat	÷	O	O	÷	÷	-
Sulphur	÷	O	O	÷	÷	-
Direct	-	-	-	÷	-	÷

Color removal: O unsatisfactory, ÷ good, s specially suitable, – not investigated

Figure 29: Summary of effectiveness of effluent treatment processes for various dyestuff classes. (Clarke and Anliker, 1980)

One of the considerations in the application of biodegradation is the toxicity and biodegradability of the resulting products. With the help of the technique in genetic engineering, this problem has found new solutions. By genetic manipulation, the microbial metabolic pathways can be altered to acquire new catabolic pathway (Timmis *et al.*, 1989). Mortlock (1982) had discussed the possibility of obtaining novel metabolic pathways through laboratory selection and illustrated with many examples. The acquisitions include various enzymatic activity involving oxidoreductase, transferase, hydrolase, isomerase and synthetase.

Microorganisms isolated for the degradative ability of natural or artificial compounds often belong to the genus *Pseudomonas*. *Pseudomonas* strains are found to have extraordinary range of catabolic pathways. Many of the catabolic pathways are encoded by a number of plasmids. These degradative plasmids are abundant in *Pseudomonas* species and the majority of them are large (>50 kb). The genetic aspect for biodegradation by *Pseudomonas* has been discussed by Haas (1983). Genetic studies of *Pseudomonas* species has opened the way for plasmid-assisted molecular breeding to enhance the emergence of novel degradative strains for persistent toxic chemicals. Kellogg *et al.* (1981) tried to breed microorganisms capable of utilizing 2,4,5-trichlorophenoxyacetic acid (a kind of synthetic herbicides) by inoculating into a chemostat microorganisms from waste-dumping sites with microorganisms harbouring a variety of degradative plasmids such as CAM, TOL, SAL, pAC21 and pAC25. Finally, bacterial strains capable of completely degrading 2,4,5-trichlorophenoxyacetic acid by using it as sole carbon source at high concentrations (>1mg/ml) were obtained.

Strain H-12 is found to harbour a plasmid of about 23 kb. It is not yet determined whether the degradative ability resides on this plasmid or chromosome. However, it is expected that through the further genetic manipulation of the degradative genes, the degradative ability can be enhanced.

REFERENCES

Alexander, M. (1981) Biodegradation of chemicals of environmental concern. *Science* 211:132-138.

Allen, R.C.M., Clark, C.O., Davis, R.R., Lapworth, M., Miles, L.W.C., Skelly, J.K., Stead, E., Wyles, J.M., Wood, W.E., Appel, W.D., Campbell, K.S., Fusser, L., Gonet, F., Habel, O.T., Inkpen, N.A., Loughlin, J.E., Martin, W.R., Paine, G.P., Schlaeppi, F., Swartzwelder, G.M., Sylvester, C.A., Watson, J.G., Wich, E.A., Beal, D.R., Cady, W.H., Howes, W.R., Manara, L.J., Tew, J.G., Turner, R.A. and Weil, H.A. (1971) Colour idex. The Society of Dyers and Colourists. Amercian Association of Textile Chemists and Colourists. Lund Humphries, Bradford.

Anliker, R. (1977) Colour chemistry and the environment. *Rev. Prog. Coloration* 8:60-72.

Anliker, R., Dürig, G., Steinle, D. and Moriconi, E.J. (1988) List of colorants to be classified as toxic. *J. Soc. Dyers Colourists* 104:223-225.

Atlas, R.M. (1981) Microbial degradation of petroleum hydrocarbons: an environmental perspective. *Microbiol. Rev.* 45:180-209.

Auletta, A.E., Kuzava, J.M. and Parmar, A.S. (1977) Lack of mutagenic activity of a series of food dyes for *Salmonella typhimurium*. *Mutat. Res.* 56:203-206.

Brown, D., Hitz, H.R. and Schäfer, L. (1981) The assessment of the possible inhibitory effect of dyestuffs on aerobic waste-water bacteria experience with a sceening test. *Chemosphere* 10:245-261.

Brown, J.A. and Weintraub, M. (1982) Biooxidation of paint process wastewater. *J. Water Pollut. Control Fed.* 54:1127-1130.

Brown, J.P. (1981) Reduction of polymeric azo and nitro dyes by intestinal bacteria. *Appl. Environ. Microbiol.* 41:1283-1286.

Brown, J.P., Roehm, G.W. and Brown, R.J. (1978) Mutagenicity testing of certified food colors and related azo, xanthene and triphenylmethane dyes with the *Salmonella*/microsome system. *Mutat. Res.* 56:249-271.

Bumpus, J. and Brock, B.J. (1988) Biodegradation of crystal violet by the white rot fungus *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* 54:1143-1150.

Chung, K., Fulk, G.E. and Andrews, A.W. (1981) Mutagenicity testing of some commonly used dyes. *Appl. Environ. Microbiol.* 42:641-648.

Chung, K., Fulk, G.E. and Egan, M. (1978) Reduction of azo dyes by intestinal anaerobes. *Appl. Environ. Microbiol.* 35:558-562.

Clarke, E.A. and Anliker, R. (1980) Organic dyes and pigments. *In*: The handbook of environmental chemistry Volume 3 Part A Anthropogenic compounds. Hutzinger, O. (ed.) pp.181-215. Spring-Verlag, Berlin.

Cook, A.M., Grossenbacher H. and Hütter, R. (1983) Isolation and cultivation of microbes with biodegradative potential. *Experientia* 39:1191-1198.

Cripps, C., Bumpus, J.A. and Aust, S.D. (1990) Biodegradation of azo and heterocyclic dyes by *Phanerochaeta chrysosporium*. *Appl. Environ. Microbiol.* 56:1114-1118.

Davis, J. (1991) Improving dye waste water treatment. *Am. Dyest. Rep.* 80(3):19.

El-Geundi, M.S. (1991) Colour removal from textile effluents by adsorption techniques. *Wat. Res.* 25:271-273.

Ensley, B.D., Ratzkin B.J., Osslund, T.D., Simon, M.J., Wackett, L.P. and Gibson, D.T. (1983) Expression of naphthalene oxidation genes in *Escherichia Coli* results in the biosynthesis of indigo. *Science* 222:167-169.

Environmental Protection Department (1989) Draft Waste Disposal Plan for Hong Kong. Hong Kong Government.

Filippov, V.M., Timofeeva, T.S., Endyus'kin, P.N., Chalykh, E.A., Shloma, E.N. and Al'bert, A.A. (1988) Purification of dye plant waste water with aniline and formaldehyde. *Khimiya i Tekhnologiya Vody (Soviet J. Wat. Chem. Technol.)* 10:47-50.

Finn, R.K. (1983) Use of specialized microbial strains in the treatment of industrial waste and in soil decontamination. *Experientia* 39:1231-1236.

Fox, M.R. and Pierce, J.H. (1990) Indigo: past and present. *Text. Chem. Color.* 22(4):13-15.

Fung, D.Y.C. and Miller, R.D. (1973) Effect of dyes on bacterial growth. *Appl. Microbiol.* 25:793-799.

Gallup, J.D. (1979) Water pollution in the textile industry. Environmental Symposium - Textile Industry and the Environment, American Association of Textile Chemists and Colorists, North Carolina.

Ghisalba, O. (1983) Chemical wastes and their biodegradation - an overview. *Experientia* 39:1247-1257.

Giri, A.K. and Mukherjee, A. (1990) Sister chromatid exchange induced by secondary and tertiary amine containing dyes and in combination with nitrite *in vivo* in mice. *Cancer Lett.* 52:33-37.

Glenn, J.K. and Gold, M.H. (1983) Decolorization of several polymeric dyes by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 45:1741-1747.

Goodson, L.A. (1979) Environmental impact - measurement and control. Environmental Symposium - Textile Industry and the Environment, American Association of Textile Chemists and Colorists, North Carolina.

Gordon, P.F. and Gregory, P. (1983) Organic chemistry in colour. Springer-Verlag, Berlin.

Grosjean, D., Whitmore, P.M., Cass G.R. and Druzik, J.R. (1988) Ozone fading of natural organic colorants: mechanisms and products of the reaction of ozone with indigos. *Environ. Sci. Technol.* 22:292-298.

Haas, D. (1983) Genetic aspects of biodegradation by pseudomonads. *Experientia* 39:1199-1213.

Hicks, R.J., Stotzky, G. and Voris, P.V. (1990) Review and evaluation of the effects of xenobiotic chemicals on microorganisms in soil. *In*: Advances in applied microbiology Volume 35. Neidleman, S.L. and Laskin, A.I. (ed.) pp.195-253. Academic Press, Inc., San Diego.

Holme, I. (1984) Ecological aspects of colour chemistry. *In*: Developments in the chemistry and technology of organic dyes. Griffiths, J. (ed.) pp.111-128. Blackwell Scientific Publications, Oxford.

Horning, R.H. (1979) Priority pollutants in dyes - their occurrance and significance. Environmental Symposium - Textile Industry and the Environment, American Association of Textile Chemists and Colorists, North Carolina.

Huang, M.T., Miwa, G.T., Cronheim, N. and Lu, A.Y.H. (1979) Rat liver cytosolic azoreductase. *J. Biol. Chem.* 254:11223-11227.

Idaka, E., Horitsu, H. and Ogawa, T. (1987a) Some properties of azoreductase produced by *Pseudomonas cepacia*. *Bull. Environ. Contam. Toxicol.* 39:982-989.

Idaka, E., Ogawa, T. and Horitsu, H. (1987b) Reductive metabolism of aminoazobenzenes by *Pseudomonas cepacia*. *Bull. Environ. Contam. Toxicol.* 39:100-107.

Idaka, E., Ogawa, T. and Horitsu, H. (1987c) Oxidative pathway after reduction of p-aminoazobenzene by *Pseudomonas cepacia*. *Bull. Environ. Contam. Toxicol.* 39:108-113.

Idaka, E., Ogawa, T., Horitsu, H. and Tomoyeda, M. (1978) Degradation of azo compounds by *Aeromonas hydrophila* var. 24B. *J. Soc. Dyers Colourists* 94:91-94.

Idaka, E., Ogawa, T., Yatome, C. and Horitsu, H. (1985) Behavior of activated sludge with dyes. *Bull. Environ. Contam. Toxicol.* 35:729-734.

Kanekar, P. and Sarnaik, S. (1991) An acivated sludge process to reduce the pollution load of a dye-industry waste. *Environ. Pollut.* 70:27-33.

Keil, H., Saint, C.M. and Williams, P.A. (1987) Gene organization of the first catabolic operon of TOL plasmid pWW53: production of indgo by the *xyl*A gene product. *J. Bacteriol.* 169:764-770.

Keith, L.H. and Telliard, W.A. (1979) Priority pollutants I-a perspective view. *Environ. Sci. Technol.* 13:416-423.

Kellogg, S.T., Chatterjee, D.K. and Chakrabarty, A.M. (1981) Plasmid-assisted molecular breeding: new technique for enhanced biodegradation of persistent toxic chemicals. *Science* 214:1133-1135.

Kobayashi, H. and Rittmann, B.E. (1982) Microbial removal of hazardous organic compounds. *Environ. Sci. Tehchnol.* 16:170A-183A.

Kulla, H.G. (1981) Aerobic bacterial degradation of azo dyes. *In*: Microbial degradation of xenobiotics and recalcitrant compounds. Leisinger, T., Cook, A.M., Hütter, R. and Nüesch, J. (ed.) pp.387-399. Academic Press, London.

Kulla, H.G., Krieg, R., Zimmermann, T. and Leisinger, T. (1984) Experimental evolution of azo dye-degrading bacteria. *In*: Current prospectives in microbial ecology. Klug, J. and Reddy, C.A. (ed.) pp.663-667. American Society for Microbiology, Washington D.C.

Kurkela, S., Lehväslaiho, H., Palva, E.T. and Teeri T.H. (1988) Cloning, nucleotide sequence and characterization of genes encoding naphthalene dioxygenase of *Pseudomonas putida* strain NCIB9816. *Gene* 73:355-362.

Kwasniewska, K. (1985) Biodegradation of crystal violet (hexamethy-p-rosaniline chloride) by oxidative red yeasts. *Bull. Environ. Contam. Toxicol.* 34:323-330.

Lee, W.N., Paris, D.F., Steen, W.C. and Baughman, G.L. (1980) Correlation of microbial degradation rates with chemical structure. *Environ. Sci. Technol.* 14:1143-1144.

Leisinger, T. (1983) Microorganisms and xenobiotic compounds. *Experientia* 39:1183-1191.

Lynn, J.E. (1985) Mode indigo. *Am. Dyest. Rep.* 74(8):9.

Manulyak, I.I., Udod, V.M., Nesynova, L.I. and Kravchuk, V.N. (1987) Anaerobic treatment of industrial waste waters of fine organic synthesis plants. *Khimiya i Tekhnologiya Vody (Soviet J. Wat. Chem. Technol.)* 9:167-169.

Mermod, N., Harayama, S. and Timmis, K.N. (1986) New route to bacterial production of indigo. *Biotechnology* 4:321-324.

Meyer, U. (1981) Biodegradation of synthetic organic colorants. *In*: Microbial degradation of xenobiotics and recalcitrant compounds. Leisinger, T., Cook, A.M., Hütter, R. and Nüesch, J. (ed.) pp.371-385. Academic Press, London.

Michaels, G.B. and Lewis, D.L. (1985) Sorption and toxicity of azo and triphenylmethane dyes to aquatic microbial populations. *Environ. Toxicol. Chem.* 4:45-50.

Mortlock, R.P. (1982) Metabolic acquisitions through laboratory selection. *Ann. Rev. Microbiol.* 36:259-284.

Netzer, A., Beszedits, S., Wilkinson, P. and Miyamoto, H. (1976) Advanced physical-chemical treatment of dye wastes. *Prog. Wat. Tech.* 8:25-37.

Ogawa, T., Idaka, E. and Yatome, C. (1981) Acclimation of activated sludge to dye. *Bull. Environ. Contam. Toxicol.* 26:31-37.

Ogawa, T., Shibata, M., Yatome, C. and Idaka, E. (1988) Growth inhibition of *Bacillus subtilis* by basic dyes. *Bull. Environ. Contam. Toxicol.* 40:545-552.

Oshima, T., Kawai, S. and Egami, F. (1965) Oxidation of indole to indigotin by *Pseudomonas indoloxidans*. *J. Biochem.* 58:259-263.

Pagga, U. and Brown, D. (1986) The degradation of dyestuffs: Part II Behaviour of dyestuffs in aerobic biodegradation tests. *Chemosphere* 15:479-491.

Painter, H.A. and King, E.F. (1985) Biodegradation of water-soluble compounds. *In*: The handbook of environmental chemistry Volume 2 Part C Reactions and processes. Hutzinger, O. (ed.) pp.87-120. Spring-Verlag, Berlin .

Patterson, J.W. and Kodukala, P.S. (1981) Biodegradation of hazardous organic pollutants. *Chem. Eng. Progr.* 77(4):48-55.

Robra, K.H. (1986) Special methods in microbial degradation. *In*: Biotechnology Volume 8 Microbial degradations. Rehm, H.-J. and Reed, G. (ed.) pp.651-670. VCH, Weinheim.

Robinson, P.K. (1989) Biotechnological involvement and advances in waste-water treatment. *In*: Resources and applications of biotechnology --- The new wave. Greenshields, R. (ed.) pp.279-297. The Macmillan Press Ltd., London.

Roychoudhury, A. and Giri, A.K. (1989) Effects of certain food dyes on chromosomes of *Allium cepa*. *Mutat. Res.* 223:313-319.

Sankaranarayanan, N. and Murthy, M.S.S. (1979) Testing of some permitted food colours for the induction of gene conversion in diploid yeast. *Mutat. Res.* 67:309-314.

Sebek, O.K. and Jäger, H. (1962) Divergent pathways of indole metabolism in *Chromobacterium violaceum*. *Nature* 196:793-795.

Shaul, G.M., Holdsworth, T.J., Dempsey, C.R. and Dostal K.A. (1991) Fate of water soluble azo dyes in the activated sludge process. *Chemosphere* 22:107-119.

So, K.O. (1989) Decolorization and biodegradation of methyl red by *Acetobacter liquefaciens*. Master Thesis. The Chinese University of Hong Kong.

Straley, J.P. (1984) Selected bacterial cultures for textile wastewaters. *Am. Dyest. Rep.* 73(9):46-57 & 73(10):34-47.

Tabak, H.H., Quave, S.A., Mashni, C.I. and Barth, E.F. (1981) Biodegradability studies with organic priority pollutant compounds. *J. Water Pollut. Control Fed.* 53:1503-1518.

Takahara, Y., Takasaki, Y. and Tanabe, O. (1961) Studies on the reduction of indigo in industrial fermentation vat (XVIII) on the growth factor of the strain no. S-8 (4). *J. Ferment. Technol.* 39:183-187.

Takahara, Y., Takasaki, Y. and Tanabe, O. (1962) Studies on the reduction of indigo in industrial fermentation vat (XX) on the reductase activity. *J. Ferment. Technol.* 40:80-84.

Takahara, Y. and Tanabe, O. (1960) Studies on the reduction of indigo in industrial fermentation vat (VII). *J. Ferment. Technol.* 38:329-331.

Takahara, Y. and Tanabe, O. (1962) Studies on the reduction of indigo in industrial fermentation vat (XIX) --- Taxonomic characteristics of strain no. S-8. *J. Ferment. Technol.* 40:77-80.

Tamura, T., Miyoshi, T., Boki, K. and Tanada, S. (1987a) Treatment of indigo carmine wastes and with granular activated carbon and its adsorption process into micropores. *Jpn. J. Hyg.* 42:858-864.

Tamura, T., Miyoshi, T., Boki, K. and Tanada, S. (1987b) Adsorption rate of indigo carmine onto activated carbon for social hygiene. *Tokushima J. Exp. Med.* 34:97-100.

Tamura, T., Miyoshi, T., Boki, K. and Tanada, S. (1988) Adsoption of indigo carmine by activated carbon. *Environ. Technol. Lett.* 9:281-286.

Tarasevich, I., Doroshenko, V.E., Rudenko, V.M. and Ivanova, Z.G. (1988) Adsorption of anionic dyes on carbon-mineral sorbents. *Khimiya i Tekhnologiya Vody (Soviet J. Wat. Chem. Technol.)* 10:315-317.

Timmis, K.N., Rojo, F. and Ramos, J.L. (1989) Design of new pathways for the catabolism of environmental pollutants. *In*: Biotechnology and biodegradation. Kamely, D., Chakrabarty, A. and Omenn, G.S. (ed.) pp.61-82. Gulf Publishing Company, Houston.

Trotman, E.R. (1970) Dyeing and chemical technology of textile fibres. Charles Griffin & Co. Ltd., London.

Urushigawa, Y. and Yonezawa, Y. (1977) Chemico-biological interactions in biological purification system II. Biodegradation of azocompounds by activated sludge. *Bull. Environ. Contam. Toxicol.* 17:214-218.

Wallnöfer, P.R. and Engelhardt, G. (1984) Aromatic and heterocyclic structures. *In*: Biotechnology Volume 6a Biotransformations. Rehm, H.-J. and Reed, G. (ed.) pp.277-328. Verlag Chemie, Weinheim.

Wang, Y., Madsen, E.L. and Alexander, M. (1985) Microbial degradation by mineralization or cometabolism determined by chemical concentration and environment. *J. Agri. Food Chem.* 33:495-499.

Wu, Y., Zhang, S.Q., Ma, G.H., Song, D.L. and Zhao, J.Y. (1989) Study on biosynthesis of indigo involving transferring naphthalene plasmid DNA from *Pseudomonas* to *E. coli*. *I Chuan Hsueh Pao* 16:318-324.

Yakovlev, S.V., Krasnoborod'ko, I.G. and Svetashova, E.S. (1988) Electrochemical decoloring of waste water. *Khimiya i Tekhnologiya Vody (Soviet J. Wat. Chem. Technol.)* 10:266-269.

Yang, X. (1990) Treatment of dyeing wastewater in China. *Text. Asia* 21(10):178-183.

Yonezawa, Y. and Urushigawa, Y. (1977) Chemico-biological interactions in biological purification system I. Growth inhibition effect of azo compounds on activated sludge microorganisms. *Bull. Environ. Contam. Toxicol.* 17:208-213.

Zimmermann, T., Gasser, F., Kulla, H.G. and Leisinger, T. (1984) Comparison of two bacterial azoreductases acquired during adaptation to growth on azo dyes. *Arch. Microbiol.* 138:37-43.

Zimmermann, T., Kulla, H.G. and Leisinger, T. (1982) Properties of purified orange II azoreductase, the enzyme initiating azo dye degradation by *Pseudomonas* KF46. *Eur. J. Biochem.* 129:197-203.